Neuroprotection by Small Molecule Activators of the Nerve Growth Factor Receptor

Bo Lin, Michael C. Pirrung, Liu Deng, Zhitao Li, Yufa Liu, and Nicholas J. G. Webster

Veterans Medical Research Foundation and Veterans Affairs San Diego Healthcare System, San Diego, California (B.L., N.J.G.W.); Department of Medicine, University of California, San Diego, La Jolla, California (N.J.G.W.); and the Department of Chemistry, University of California, Riverside, California (M.C.P., L.D., Z.L., Y.L.)

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ABSTRACT

There is a great deal of interest in neurotrophin therapy to prevent neuronal degeneration. However, the blood-brain barrier presents a major hurdle in the use of peptide therapeutics. The goal of this study was to identify small molecule, cell-permeable nerve growth factor (NGF) activators. Combinatorial libraries of asterriquinones (>300) and mono-indolyl-quinones (>60) were screened using a 96-well enzyme-linked immunosorbent assay that detects phosphorylated TrkA, the NGF receptor. The libraries were also screened for dose-dependent cytotoxicity. From these screens, we generated quantitative structure-activity relationship models for activity and toxicity, and then we selected two compounds, 2-(6-chloro-1H-indol-3-yl)-5-(2-cyclopropyl-1H-indol-3-yl)-3,6-dihydroxy-[1,4]benzoquinone (1H5) and 2,5-dimethoxy-3-(7-fluoro-1H-indol-3-yl)-[1,4]-benzoquinone (5E5), for further study based on high activity and low toxicity. Compound 1H5 (30 μM) is an asterriquinone that is a moderate TrkA activator (50% the activity of 100 ng/ml NGF), and it shows little toxicity at concentrations up to 100 μM. 1H5 can protect differentiated PC12 neurons from apoptotic cell death induced by NGF withdrawal. Compound 5E5 (30 μM) is a mono-indolyl-quinone that is a very strong activator of TrkA (>200% the activity of 100 ng/ml NGF), and it is nontoxic at concentrations up to 10 μM. Activation of TrkA can be detected at 1 μM 5E5, and 3 to 10 μM 5E5 activates TrkA and extracellular signal-regulated kinase as strongly as a maximal dose of NGF (100 ng/ml). A combination of a low dose of 5E5 (1 μM) with a submaximal dose of NGF (10 ng/ml) promotes neuronal differentiation of PC12 cells. These compounds represent a new class of TrkA activators that could have potential utility in the treatment of neurodegenerative diseases.

Nerve growth factor (NGF) belongs to a family of structurally related neurotrophin proteins, including BDNF, NT-3, and NT-4/5 that function to support the growth and survival of many populations of neurons (Friedman and Greene, 1999; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001).

NGF binds the TrkA receptor, whereas BDNF and NT-4/5 bind the TrkB receptor, and NT-3 primarily binds the TrkC receptor (Patapoutian and Reichardt, 2001). These Trk neurotrophin receptors are able to protect neuronal cells from apoptosis, and they can stimulate neuronal regeneration in model systems (Bibel and Barde, 2000; Thoenen, 2000; Huang and Reichardt, 2001; Poo, 2001). Hence, there is a great deal of interest in whether NGF and other neurotrophins can prevent or reverse cognitive decline, neurodegenerative diseases, or promote spinal cord repair (Winkler et al., 1998; Horner and Gage, 2000). A complication is that neurotrophins also bind to the p75NTR, a member of the tumor necrosis factor receptor superfamily (Bothwell, 1995; Dechant and Barde, 2002). Activation of the p75NTR causes cell death rather than survival, and p75NTR−/− mice show reductions in neuronal cell death after pilocarpine-induced status.

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ABBREVIATIONS: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; DAQ-B1, demethylasterriquinone-B1, 2-[2-(1,1-dimethyl-allyl)-1H-indol-3-yl]-3,6-dihydroxy-5-[7-(3-methyl-but-2-enyl)-1H-indol-3-yl]-[1,4]benzoquinone; IR, insulin receptor; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; SFM, serum-free medium; QSAR, quantitative structure-activity relationship; RMS, root mean square; CV, coefficient of variation; 1H5, 2-(6-chloro-1H-indol-3-yl)-3,6-dihydroxy-[1,4]benzoquinone; 5E5, 2,5-dimethoxy-3-(7-fluoro-1H-indol-3-yl)-[1,4]-benzoquinone; MAPK, mitogen-activated protein kinase; PI3-kinase, phosphatidylinositol 3-kinase.
seizures compared with wild-type mice (Troy et al., 2002). Therefore, the final effect of a neurotrophin is a balance between the cell survival signal derived from the Trk receptor family and the cell death signal from the p75NTR. Indeed, each neurotrophin causes cell death in the ~30% of cultured hippocampal neurons expressing p75 but lacking the cognate Trk receptor (Friedman, 2000).

Preclinical and clinical findings suggest that neurotrophins are a promising therapy for peripheral neuropathies (McMahon and Priestly, 1995) and neurodegenerative diseases, such as Alzheimer’s (Sivanathan and Leavitt, 2005; Tuszynski et al., 2005) and Parkinson’s disease (Shime and Chiba, 2001). However, neurotrophins do not make good drug candidates because of their poor pharmacokinetic behavior and bioavailability at the desired targets. One of the major hurdles for neurotrophin therapy is the lack of passage of peptide hormones across the blood-brain barrier (Thorne and Frey, 2001; Miller, 2002). Peripheral administration of peptide hormones only leads to a small increase in their intracerebral concentration. This has necessitated complicated methods of delivery such as via the olfactory neural pathway (Chen et al., 1998), ex vivo gene therapy by intracranial injection of NGF-expressing fibroblasts (Tuszynski et al., 2002), or placement of indwelling catheters to allow neurotrophin infusion. Therefore, much effort has been devoted to the search for nonpeptidyl small molecule neurotrophin mimics, which elicit the desired neuroregenerative responses of neurotrophins (Saragovi and Gehring, 2000; Dago et al., 2002).

Asterriquinones are naturally occurring bis-indolyl-dihydroxyquinones that activate tyrosine kinase receptors. They were originally identified as activators of the insulin receptor (Zhang et al., 1999; Liu et al., 2000). The molecules are small and readily cell-permeable, and they act directly on the receptor tyrosine kinase domain, although the mechanism is not known (Zhang et al., 1999). The original compound demethylasterriquinone-B1 (DAQ-B1) was demonstrated to cross the blood-brain barrier, and, in a separate study, it was shown to activate the NGF receptor, although it was toxic to neurons (Wilkie et al., 2001; Air et al., 2002). Therefore, we hypothesized that similar compounds could potentially activate signaling in the central nervous system and be used as oral NGF activators for neurotrophin therapy. These compounds would have the additional, very important advantage that by targeting the kinase domain of TrkA, it may be possible to avoid deleterious effects due to the activation of the p75NTR receptor, which lacks the tyrosine kinase domain. In this study, we used DAQ-B1 as a lead compound and screened a library of closely related structures to identify nontoxic and specific NGF receptor activators that have neuroprotective properties.

Materials and Methods

Materials. Rat PC12 pheochromocytoma cells were purchased from American Type Culture Collection (Manassas, VA); PC12-TrkA (clone624) cells were kindly provided by Dr. W. C. Mobley (Stanford University Medical Center, Stanford, CA); NIH3T3-TrkA, TrkB, and TrkC cells were kindly provided by Dr. D. R. Kaplan (University of Toronto, Toronto, ON, Canada). Rabbit polyclonal anti-phospho-TrkA (Tyr490), anti-phospho-TrkA (Tyr747/755), and anti-phospho-Akt (Ser473) were purchased from Cell Signaling Technology Inc. (Danvers, MA). Rabbit anti-IR/insulin-like growth factor-1 receptor (pY1162/1163) was from BioSource International (Camarillo, CA). Mouse anti-phospho-extracellular signal-regulated kinase (ERK) (Tyr204) and rabbit polyclonal anti-TrkA (SC-11) and rabbit anti-IRβ (SC-711) and horseradish peroxidase-linked anti-rabbit or anti-mouse antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine (PY20) antibodies were from BD Biosciences Transduction Laboratories (San Diego, CA). The p13suc1 agarose conjugate was purchased from Upstate Biotechnology (Lake Placid, NY). PP1 was from Alexis Laboratories (San Diego, CA). Cell culture medium and fetal bovine and horse serum were purchased from Invitrogen (Carlsbad, CA). 2.5S NGF was purchased from Roche (Pleasanton, CA). All other reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cell Culture. PC12 cells were grown in Ham’s F-12K medium supplemented with 5% fetal bovine serum and 15% horse serum and antibiotics at 37 °C in a 5% CO2 environment. PC12-TrkA (clone624) cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/l glucose) supplemented with 10% fetal bovine serum and 2.5% horse serum and 200 μg/ml Geneticin (G-418; Invitrogen) and antibiotics at 37 °C in a 7.5% CO2 environment. CHO-TrkA (clone77) cells and CHO-IRβ cells were grown in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 500 μg/ml G-418 and antibiotics at 37 °C in a 7.5% CO2 environment. NIH3T3-TrkA, TrkB, and TrkC cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/l glucose) medium supplemented with 10% bovine calf serum and 200 μg/ml G-418 and antibiotics at 37 °C in a 5% CO2 environment.

A 96-Well ELISA for Library Screening. CHO-TrkA cells (1 × 104/well) were rendered quiescent by serum starvation in Ham’s F-12 plus 0.1% bovine serum albumin for 48 h in 96-well plates and then stimulated with compounds for 10 min at 37 °C. Compounds were dissolved in dimethyl sulfoxide and used at a final concentration of 30 μM in Krebs-Ringer phosphate-HEPES supplemented with 200 μg/ml ascorbic acid. Control wells received 100 ng/ml 2.5S NGF or dimethyl sulfoxide vehicle. Each treatment was performed in triplicate. Cells were lysed with 50 μl of modified radioimmunoprecipitation assay buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 10 mM EDTA, 0.1% SDS, 1 mM Na3VO4, 160 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μM leupeptin) for 20 min, and then the whole extract (50 μl) was transferred to a microtiter plate coated with anti-phosphotyrosine antibody PY20. The extract was incubated on the plate overnight at 4 °C, and then the captured proteins were washed with 0.1% Tween 20-phosphate-buffered saline buffer three times. The immobilized proteins were probed with anti-TrkA antibodies and detected by anti-rabbit-IgG-horseradish peroxidase using a colorimetric assay with tetramethylbenzidine as substrate. The data are presented as percentage of activity relative to the 100 ng/ml NGF.

Western Blotting. Cells (1 × 105/well) were starved for 48 h in six-well plates, and then the cells were stimulated with compounds or NGF for 10 min, washed with ice-cold phosphate-buffered saline, lysed with 100 μl of SDS sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue, and 100 mM dithiothreitol), boiled for 10 min to denature proteins, and sonicated for 15 min to shear the chromosomal DNA. Equal volumes (30 μl) of lysate were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels, and they were electroblotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Bedford, MA). The membranes were blocked with 5% nonfat dried milk in TBS-Tween (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 60 min at room temperature. Blots were incubated with primary antibodies in blocking buffer overnight at 4 °C, washed three times in TBS-Tween, and then they were incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescent detection. Anti-phospho-TrkA (Tyr490), anti-phospho-TrkA (Tyr747/755), anti-phospho-Akt (Ser473), anti-phospho-ERK1 (Tyr204), and anti-phosphotyrosine (PY20) antibodies were used at a dilution of...
1:1000. To verify protein loading, the polyvinylidene difluoride membranes were immediately stripped with stripping buffer (0.5 M NaCl and 0.5 M acetic acid) for 30 min at room temperature. The membrane was then washed once for 10 min in TBS-Tween, reblocked, and blotted with antibodies to internal control protein-ERK1. The intensities of the bands were quantified using National Institutes of Health ImageJ (http://rsb.info.nih.gov/ij/) with an Agfa Arcus II scanner (Agfa-Gevaert, Montsel, Belgium).

**Cell Viability.** After the cells (5 × 10^5/well) were cultured for 72 h with increasing concentrations of compounds (3, 10, 30, and 100 μM), the number of viable cells was determined by the MTS/NADH colorimetric viability assay (Promega, Madison, WI). In brief, 20 μl of freshly prepared MTS/phenoxyethanol solution was added to each well, and the absorbance at 490 nm was recorded after incubating at 37 °C in the humidified 5% CO₂ chamber for 1 to 4 h.

**PC12 Survival Assay.** Estimation of the survival of differentiated PC12 cells, deprived of serum and NGF, was carried out as described previously (Gronborg et al., 1999) with minor modifications. In brief, 1 × 10⁶ PC12 cells were seeded in each well of a 96-well plate and differentiated in the presence of serum and 100 ng/ml 2.5S NGF for 7 days. The medium was replaced with serum-free Ham’s F-12K medium (SFM) supplemented with compounds at the indicated concentrations. NGF (100 ng/ml) was included as a positive control. The number of viable cells was evaluated at 24, 48, and 72 h by measurement of the ability of the cells to reduce the tetrazolium compound MTS (Promega) according to instructions given by the manufacturer.

**Stimulation of Neurite Outgrowth in PC12 Cells.** PC12 cells were seeded in poly-L-lysine-coated six-well plates (2 × 10⁶/well) in growth medium. The next day, cells were stimulated with 5E5, NGF, 5E5 plus NGF in normal medium for 4 days, changing medium with treatments every 2 days. For each well, approximately 10 images were acquired randomly by scanning the wells from left to right and top to bottom. For calculation of percentage of neurite-bearing cells, cells were considered to be neurite-bearing when the neurite length was >1.5 × cell body diameter (Morooka and Nishida, 1998). For neurite length measurements, the lengths of the individual neurites for each cell were measured using the ImageJ software. Length was defined as the straight-line distance from the tip of the neurite to the junction between the cell body and neurite base. In branched neurites, the length of the longest branch was measured from the tip of the neurite to the cell body and then each branch was measured from the tip of the neurite to the neurite branch point (Schmidt et al., 1997). In each instance, eight random fields (100×) were scored (total of 1500 cells) for neurite outgrowth.

**Quantitative Structure-Activity Relationship Studies.** Using the TSAR 3.0 suite of programs (Accelrys, San Diego, CA), QSAR studies were performed on the original data set of 334-molecule asterriquinone library and 62-molecule mono-indolyl-quinone library. For the asterriquinone library, 14 substituents on the core indolequinone structure of DAQ-B1 were considered (Fig. 2A). Thirteen independent variables for each substituent were incorporated into the initial QSAR model, including molecular refractivity (aromatic), Verloop steric parameters (L, B1, B2, B3, and B4), molecular mass, molecular surface area, molecular volume, ellipsoidal volume, Moriguchi octanol-water partition coefficient, Weiser coefficient (Kovatcheva et al., 2003). We also included six whole-molecule descriptors, including molecular mass, molecular volume, ellipsoidal volume, total dipole moment, log P, and molecular refractivity. Due to the rotational symmetry of the parent molecule (the indoles are equivalent), the library was numbered in both directions and combined. For the mono-indolyl-quinone library, eight substituents were considered. Twenty-five initial independent variables were considered for each substituent, including PI (aromatic), MR (aromatic), Swain and Lupton F, Swain and Lupton R, Sigma Meta, Sigma Para, Taft ES, Verloop steric parameters (L, B1, B2, B3, B4, and B5), molecular mass, molecular surface area, molecular volume, moment of inertia (size 1, 2, 3; length 1, 2, 3), ellipsoidal volume, Moriguchi octanol-water partition coefficient, and Weiser coefficient. Since the monoquinone library was much smaller than the asterriquinone library, we used data reduction techniques to eliminate variables with high correlation. Correlation coefficients were generated between all potential variables. Variables with correlation coefficients greater than 0.90 were considered redundant, and all but one were eliminated. This data reduction resulted in 45 variables with reduced collinearity.

Forward feed neural networks were generated using the TrkA activity or toxicity as target parameter and the substituent or whole molecule parameters as variables. The ratio of variables to samples was approximately 1:2 to prevent overfitting of the data. After convergence of the data, the model-predicted values were plotted against the empirical values, and correlation coefficients were generated. The best RMS fit of the model was compared with the test RMS fit to gauge the predictive value of the model. The parameter dependencies were determined by systematically varying each parameter and by measuring the effect on the output variable.

Multiple regression models were generated using F-stepping. Initially, models were limited to linear regression. Leave-out-groups-of-three cross-validation with varied random selections was used to test predictive power (r²(CV)). The F-enter and F-leave parameters were systematically varied to optimize the model without including too many parameters. Partial F values were inspected to look for obvious cut-offs. After running linear regression models, the models were rerun but allowing the parameters to vary up to the fourth power. The predicted values were correlated against the empirical values.

**Statistical Analysis.** Biological data were analyzed by analysis of variance or t test in Microsoft Excel 2000 (Microsoft, Redmond, WA). Results are presented as mean and standard deviation or standard error. A two-tailed P value < 0.05 was considered significant.

**Results**

**Screening Combinatorial Libraries for Activation of TrkA.** The lead compound DAQ-B1 that was identified as an...
activator of the insulin receptor has a pseudo-symmetrical bis-indolylquinone structure (Fig. 1A). We synthesized a library of asterriquinones using a combinatorial approach (Fig. 1B). Details of the synthetic route have been published previously (Pirrung et al., 2005b). Aliphatic, aromatic, and halogen substitutions were included at different positions on the indole rings and the hydroxyl of the quinone ring, generating 14 possible substitution positions (Fig. 2A). This library of 334 compounds was tested for activation of the TrkA using an ELISA assay that detects phosphorylated TrkA. Briefly, all tyrosine-phosphorylated proteins were captured by PY20 antibody coated on microtiter plates. The presence of TrkA among the phosphorylated proteins was then detected by anti-TrkA antibodies. Activation of TrkA was compared with stimulation by a maximal concentration of NGF (100 ng/ml), and activation is presented as percentage of NGF. In this assay, the original compound DAQ-B1 activated TrkA to 61% of the effect of NGF, and 91 other compounds had activity >25% NGF (see Supplemental Material 1). As an index of the assay, the mean SD of the total assay for all compounds is approximately ±20%. This data set was used to generate QSAR models for activation of TrkA. We derived forward feed

Fig. 2. QSAR models for TrkA activation by both asterriquinone and mono-indolyl-quinone libraries. Compounds were used at 30 μM for the library screen. A, generic structure of the asterriquinone library. B, forward feed neural net model for TrkA activation by the asterriquinone library. Neural network was trained using a set of 188 exploratory variables with TrkA activation measured by ELISA as the target variable. Final model was obtained after 3482 cycles. C, multiple linear regression model for TrkA activation by the asterriquinone library using 188 exploratory variables. D, schematic of results from modeling of asterriquinone data. E, generic structure of the mono-indolyl-quinone library. F, forward feed neural net model for TrkA activation of mono-indolyl-quinone library. Neural network was trained using 45 exploratory variables. Final model was obtained after 1351 cycles. G, multiple linear regression model for TrkA activation by the mono-indolyl-quinone library using the 45 exploratory variables. H, schematic of results from modeling of monoquinone data. In B, C, F, and G, the actual values are shown on x-axis, and the predicted values are shown on the y-axis. Activity is relative to 100 ng/ml NGF. In D and H, bottom right semicircles depict results from forward feed neural nets, and top left semicircles depict results from multiple linear regression. Black indicates a positive correlation with the substituent parameter, and gray indicates a negative correlation.
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neural networks for activation of TrkA. Thirteen parameters were considered for each of the 14 substituents as well as six whole-molecule parameters, for a total of 188 variables for modeling. Initial models were unsuccessful. We suspected that the rotational symmetry of the molecule was limiting the power of the model (substituent 2 is equivalent to substituent 8, etc.), so we duplicated the library but we labeled the substituents in the opposite direction and combined the two. The resulting neural net had a best RMS fit of 0.098 after 3482 cycles of training. This is very close to the test RMS fit (0.103), which indicates good predictive power. The plot of predicted TrkA activation versus observed activation is shown (Fig. 2B). The model gave a good fit to the data \( r^2 = 0.53 \), explaining 53% of the variation in activity. 

The model dependencies were inspected by varying each parameter in turn. Symmetrical substituents 2/8 and 6/12 contributed six of the eight top dependencies. The eight parameters that showed the greatest dependence are shown in the Supplemental Material 1. The data were also modeled using multiple regression analysis. The same 188 independent variables were considered, and 27 variables were included in the final linear regression model using F-test stepping (F to enter = 2; F to leave = 2). Cross-validation leaving out each of 10 groups in turn was performed to test predictive power. The resulting model was highly significant (Fig. 2C), showed good correlation, explained approximately 40% of the variance \( r^2 = 0.39 \), and it had reasonable predictive power \( r^2(CV) = 0.30 \). Nonlinear regression models allowing parameters to vary up to the fourth power did not improve the fit. The 10 most significant parameters include Verloop B4 on substituents 2/8, Verloop B1 on substituents 10/4, ellipsoidal volume of substituent 9, molecular volume on substituents 11/5, total dipole moment on substituent 3, and molecular mass or log P for the whole molecule (Supplemental Material 2). As expected, the regression model included similar parameters on symmetrically related positions, e.g., 2 and 8, 4 and 10, and 5 and 11. The results of the modeling of asterriquinone activation data are depicted in schematic form in Fig. 2D. The QSAR models suggest that substituents 2, 8, and 11 on the indole ring are critical for the ability to activate TrkA as they are found by both modeling approaches.

We also tested 62 mono-indolyl-quinone intermediates against TrkA (Pirrung et al., 2005a). The generic structure is shown in Fig. 2D. Many of the mono-indolyl-quinones also activated TrkA (Supplemental Material 2), and we used QSAR to model the activity of the mono-indolyl-quinones. Eight positions of substitution were considered. Due to the smaller number of compounds in this library, we could not use all 200 substituent variables for the QSAR modeling. Therefore, we used a data reduction method to eliminate variables that are highly correlated, resulting in 45 final variables with reduced colinearity. The forward feed neural network model explained approximately 46% of the variance in activity \( r^2 = 0.46 \) (Fig. 2E). The best RMS fit was 0.038 after 1351 cycles of training compared with the test RMS fit of 0.22, indicating reasonable predictive power. The major dependencies were analyzed. The PI on substituent 7 and molecular refractivity and \( \sigma_r \) on substituent 6 showed the greatest dependencies (Supplemental Material 3). The data were also modeled using multiple regression analysis. The 45 independent variables were considered, and 10 variables were included in the final linear regression model using F-test stepping (F to enter = 1; F to leave = 1). Cross-validation by leaving out each of three groups in turn was performed to test predictive power. The resulting model for TrkA activation shows a high correlation \( r^2 = 0.90 \) between predicted and measured activity and explains 80% of the variance in the data, and it is very predictive \( r^2(CV) = 1 \) (Fig. 2F). The significant individual terms include PI on substituent 7, Swain and Lupton R on substituent 5, molecular mass or Taft ES on substituent 4, and Weiner coefficient on substituent 6 (Supplemental Material 4). The results of the modeling of monoquinone activation data are shown in schematic form in Fig. 2H. These models point to the 4, 5, and 6 substituents on the indole ring and the 7 substituent on the quinone ring being important for mono-indolyl-quinone activation of TrkA.

Screening Combinatorial Libraries for Toxicity. We also measured the toxicity of the library compounds against CHO-TrkA cells using MTS nonradioactive cell proliferation assay to measure cell viability (Supplemental Material 1 and 2). Increasing concentrations of each compound were tested (3, 10, 30, and 100 \( \mu \)M). Many compounds showed a dose-dependent toxicity with decreased cell viability. The 4-point dose-response curves were used to calculate IC\(_{50}\) values. Compounds that did not show dose-dependent cytotoxicity over the concentration range tested were arbitrarily assigned IC\(_{50}\) values of 300 \( \mu \)M. Activation of TrkA did not correlate with IC\(_{50}\) for toxicity or cell viability at 100 \( \mu \)M compound, demonstrating that the toxicity is not activity dependent (Fig. 3, A and B).

The cell viability data were also modeled by QSAR. The same 188 parameters were considered for the asterriquinone library as described above. The resulting neural network had a best RMS fit of 0.137 after 2573 cycles of training (Fig. 3C). The model showed a good correlation between predicted and actual viability data \( r^2 = 0.54 \). Analysis of the dependencies did not reveal any individual parameter that contributed dominantly to the model (data not shown). Multiple linear regression analysis generated a model containing 21 variables that explained 46% of the variance in the data with good predictive power \( r^2 = 0.46; r^2(CV) = 0.37 \) (Fig. 3D). The results of modeling the asterriquinone cell viability data are shown schematically in Fig. 3G. The most significant individual terms in this model are molecular volume of the whole molecule, ellipsoidal volume on substituents 4/10 and 6/12, Verloop L steric parameter on substituent 6/12, and aromatic molecular refractivity on substituent 2/8. According to this model, viability is favored by large volume substituents 4/10, 6/12, and 2/8 on the indole ring (Supplemental Material 3). These predictors are different from the predictors of TrkA activation, which depend on steric parameters, in agreement with the lack of dependence of TrkA activation and toxicity. Interestingly, substituents on the 2/8 positions also favor TrkA activation, suggesting that this may provide a useful site for further structural modifications.

Similar QSAR models were generated for the mono-indolyl-quinone library using the reduced set of 45 parameters. The resulting neural network had a best RMS fit of 0.148 after 849 cycles of training and showed a good correlation \( r^2 = 0.47 \) (Fig. 3E). As we had observed for the asterriquinone library, analysis of the dependencies did not reveal any dominant variables in this model. Multiple linear regression
analysis generated a model containing 14 variables and showed good predictive power ($r^2 = 0.69$, $r^2(\text{CV}) = 1$) (Fig. 3F). The most significant individual terms in this model are PI on substituent 7, Verloop L steric parameter or Taft ES on substituent 6, and $\sigma_p$ on substituent 5 (Supplemental Material 4). The results of modeling the monoquinone cell viability data are shown schematically in Fig. 3G.

**Selection of TrkA Activators and Confirmation of Activity.** We selected 1H5 from the asterriquinone library and another compound, 5E5, from the mono-indolyl-quinone library for further study based on good NGF activator activity and low toxicity. 1H5 contains a chlorine substitution on one indole group and a cyclopropyl group on the other indole (Fig. 4A). The latter group is particularly interesting because it would be predicted to force the indole and the quinone rings to be orthogonal due to steric hindrance (Fig. 4B). The monoquinone 5E5 contains a fluorine substitution at the 7-position of the indole ring and 2-methoxy substitutions on the quinone ring (Fig. 4A). The 2-methoxy groups on the quinone ring increases the dihedral angle between the indole and quinone similar to the 2-substitution on the indole (Fig. 4C). The ability of these two compounds to activate TrkA is shown in Fig. 4D. 1H5 has approximately 50% the effect of NGF, whereas 5E5 has approximately 200% the effect of a maximal dose of NGF. The dose-dependent cytotoxicity of these compounds is shown in Fig. 4E. The IC$_{50}$ for the original com-
compound DAQ-B1 is 5 μM. The monoquinone 5E5 is less toxic with an IC₅₀ of 29 μM. The asterriquinone 1H5 was nontoxic up to 30 μM, but it started to show toxicity at 100 μM.

**The Asterriquinone 1H5 Can Protect Differentiated PC12 Neurons from Apoptosis Induced by NGF and Serum Withdrawal.** We used PC12 pheochromocytoma cells to test the neurotrophic properties of the selected compounds. These cells undergo NGF-dependent neuronal differentiation, and the differentiated neurons require NGF for continued cell survival. The ability of the asterriquinone 1H5 to activate TrkA in these cells was confirmed by immunoblotting with antibodies to specific tyrosine phosphorylation sites. 1H5 caused a dose-dependent increase in phosphorylation of tyrosine 490 on TrkA and also activated ERK and Akt downstream of the receptor (Fig. 5A). We used PC12 cells that stably express TrkA to maximize our ability to detect TrkA activation. The activator activity in PC12-TrkA cells (45% the activity of 100 ng/ml NGF at 30 μM) was similar to the CHO-TrkA cells used for the library screen. Similar results were obtained in the parental PC12 cells (data not shown). To determine whether activator-induced TrkA activation was capable of stimulating a neurotrophic biological response, the asterriquinone 1H5 was tested in a neuronal survival assay. Briefly, the parental PC12 cells were differentiated in complete medium with 100 ng/ml NGF for 7 days, and then the cells were changed to SFM with or without TrkA activators. Cell viability was determined at 24, 48, and 72 h using the mitochondrial dye MTS (Fig. 5B). At 72 h, viability was 31 ± 6% in control PC12 cells, 42 ± 5% in cells treated with 30 μM 1H5, and 70 ± 4% in cells treated with 100 ng/ml NGF alone. Data are presented as percentage of the positive control cells treated with 100 ng/ml NGF in complete medium. Cell survival was not improved at 100 μM, because the activators start to be cytotoxic at that dose (data not shown). Under the microscope, most of the negative control cells (SFM only) showed multiple apoptotic figures, broken cells, and cellular debris. Treatment with NGF preserved cell morphology, and neurites can be seen on many cells. Treatment with activator 1H5 preserved the morphology of the cell bodies and prevented apoptotic figures, but there is a decrease in the number of neurites on the cells (Fig. 5C).

**The Monoquinone 5E5 Has Additive Effects with NGF to Promote Neuronal Differentiation.** The library screen indicated that 5E5 was a strong activator of TrkA in CHO-TrkA cells. Therefore, we determined the full dose-response curve for activation of TrkA and downstream signaling by 5E5 in PC12-TrkA cells. Phosphorylation of TrkA on tyrosines 490 and 674/675 is observed with 1 μM 5E5 and increases according to the doses (Fig. 6, A and B). Phosphorylation of ERK (Tyr204) (Fig. 6C) and Akt (Ser473) (Fig. 6D)
In this study, we developed combinatorial libraries of asterriquinones and screened them against TrkA receptor. We screened 334 asterriquinones and 62 mono-indolyl-quinones, and we identified 27 asterriquinones and six mono-indolyl-quinones as TrkA activators. We developed QSAR models for parallels that of the receptor, demonstrating that 5E5 activates NGF signaling pathways leading to MAPK and PI3-kinase activation. Maximal activation of PI3-kinase/Akt, however, is consistently weaker with 5E5 than NGF, but ERK activation is comparable. 5E5 caused phosphorylation of FRS2/SNT, a differentiation-specific target of NGF in neurons and PC12 cells (2-fold versus NGF) (Fig. 6E). The time course of stimulation, however, of the TrkA receptors by 5E5 and NGF is different. NGF stimulation leads to the rapid and prolonged activation of TrkA phosphorylation (>4 h); 5E5 stimulation leads to the same rapid activation of TrkA, but phosphorylation is decreased to basal levels over 60 min (Fig. 6F). This may have important implications for the ability of these activators to stimulate neuronal differentiation as prolonged activation of signaling is required. We also compared the specificity of activating Trk receptors in NIH3T3-TrkA or TrkB or TrkC cells. 5E5 was a weaker activator of TrkB (12% the activity of 100 ng/ml BDNF at 30 μM) or TrkC (43% the activity of 100 ng/ml NT-3 at 30 μM) receptors, suggesting that 5E5 is more selective for TrkA (209% the activity of 100 ng/ml NGF at 30 μM) (Fig. 6, G–I). Previous results have shown an indirect method for activation of the Trk receptor through Src family members that is inhibited by the Src inhibitor-PP1 (Lee and Chao, 2001). To rule out indirect activation of TrkA, PC12-Trk cells were pretreated with PP1 for 30 min, and then they were stimulated with 30 μM 5E5 or 100 ng/ml NGF for 10 min or with 100 μM adenosine for 2 h. Results indicated that phosphorylation of Trk receptor induced by 5E5 or NGF is insensitive to PP1 but that phosphorylation induced by adenosine is blocked (Fig. 6D). Because DAQ-B1 was first identified as an insulin receptor activator, we tested the ability of our selected compounds to activate the insulin receptor in CHO-IR3 cells. Interestingly, asterriquinone 1H5, which is most closely related to DAQ-B1, also activated the IR, but the monoquinone 5E5 showed no ability to activate the IR (Fig. 6K).

To determine whether 5E5-induced TrkA activation was capable of stimulating a neurotrophic response, we tested the ability of 5E5 to cause neuronal differentiation. PC12 cells were stimulated with complete medium plus 100 ng/ml NGF or 10 μM 5E5 (maximal nontoxic dose) for 4 days. 5E5 was unable to support neuronal differentiation under these conditions (data not shown). We then tested whether submaximal doses of both NGF (10 ng/ml) and 5E5 (1 μM) could support differentiation. Neurite length was measured, and percentage of neurite-bearing cells was calculated. As before, 5E5 alone did not support differentiation. A low dose of 10 ng/ml NGF alone was partially able to support differentiation (12% neurite-positive cells) compared with a maximal dose of 100 ng/ml NGF (22% neurite-positive cells). Similar effects were observed when counting the number of neurite-bearing cells and the length of individual neurites (Fig. 7, A and B). The addition of 1 μM 5E5 to the low dose of NGF restored maximal differentiation (Fig. 7C). These findings demonstrate that the TrkA activator can potentiate the effect of low doses of NGF and may improve neuronal differentiation.

Discussion

In this study, we developed combinatorial libraries of asterriquinones and screened them against TrkA receptor. We screened 334 asterriquinones and 62 mono-indolyl-quinones, and we identified 27 asterriquinones and six mono-indolyl-quinones as TrkA activators. We developed QSAR models for
activation of the TrkA receptor based on forward feed neural networks and multiple regression analysis. Both methods gave models that are predictive of TrkA activator activity; the asterriquinone models explain approximately 40 to 50% of the variance, and the monoquinone models explain approximately 50 to 80% of the variance. The difference in the explained variance may be the result of fewer possible substituents in the mono-indolyl-quinone structure (eight positions) compared with the asterriquinone structure (14 positions) as the number of possible structures increases.

Fig. 6. Effect of compound 5E5 on TrkA signaling. A to D, starved PC12-TrkA cells were stimulated with increasing doses of 5E5 (0.3, 1, 3, 10, 30, and 100 μM) or 100 ng/ml NGF for 10 min, lysed, and immunoblotted with anti-phospho-TrkA (Tyr490) (A), anti-phospho-TrkA (Tyr674/675) (B), anti-phospho-ERK (Tyr204) (C), and anti-phospho-Akt (Ser473) (D). Representative blots are shown, and blots were quantified with ImageJ, normalized to total ERK1/2 as a loading control. Data are mean ± S.D. of triplicate experiments, and they are presented as percentage of activity relative to 100 ng/ml NGF treatment. E, PC12-TrkA cells were stimulated with 30 μM 5E5 or 100 ng/ml NGF for 10 min, lysed, and SNT/FRS1 was precipitated on p13suc1 agarose beads. The resulting pellets were subjected to Western blot analysis with PY20 antibody. F, PC12-TrkA cells were left unstimulated (basal) or were treated with either 100 ng/ml NGF or 10 μM 5E5 for the time indicated. Cells were lysed and immunoblotted with the anti-phospho-TrkA (Tyr490). G to I, NIH3T3-TrkA, TrkB, and TrkC cells were stimulated with increasing doses of 5E5 or the corresponding neurotrophins. Cells were lysed and immunoblotted with anti-phospho-Trk (Tyr490). This antibody can recognize TrkA, TrkB, and TrkC when they are phosphorylated at the equivalent site. Representative blots are shown, and multiple blots were quantified with ImageJ. Data are mean ± S.D. of triplicate or duplicate experiments, and they are presented as percentage of activity relative to maximum neurotrophin treatment (100 ng/ml NGF or BDNF or NT-3). J, effect of PP1 on 5E5 signaling. PC12-Trk cells were pretreated with PP1 (100 nM, + or 1 μM, ++ ) for 30 min, and then they were stimulated with 30 μM 5E5 or 100 ng/ml NGF for 10 min or with 100 μM adenosine for 2 h. Cell extracts were blotted for phospho-TrkA (Tyr490), and then they were reblotted with TrkA. K, CHO-IR cells were starved and stimulated with indicated treatments for 10 min, lysed, and immunoblotted with anti-IR/insulin-like growth factor-1 receptor (pYpY1162/1163) and reblotted with anti-IRβ.
exponentially with number of positions (n^8 versus n^{14/2}
structures were, n is the number of possible substituents at
each position). Hence, we may not have enough asterriqui-
nones to derive better models. Models from both the aster-
riquinones and mono-indolyl-quinones highlight the impor-
tance of substitutions at critical residues for activation of
TrkA.

Based on the current models, a bulky substituent on the
2-position of the indole favors TrkA activation, and it also
reduces toxicity. This position is critical, because these sub-
stitutions would be expected to twist the indole group out of
the plane of the quinone due to steric hindrance between the
quinone oxygen and hydroxyl, and C-2 and C-4 of the indole
group. Molecular modeling of C-2-substituted asterriquino-
ones and mono-indolyl-quinones indicates a dihedral angle of
75 and 90° between the quinone and indole groups (Fig. 4, B
and C). Dihedral angles in compounds lacking a C-2 substitu-
tant are typically 35° due to the smaller size of the hydrogen
atom. Asterriquinones have been proposed to have cytotoxic
effects due to their ability to intercalate into DNA. The abil-
ity to intercalate depends on the coplanarity of the aromatic
groups, so a reasonable explanation for the decreased toxicity
of 2-substituted asterriquinones is the orthogonal orientation
of the indole with respect to the quinone prevents intercala-
tion. These activity and toxicity models may thus prove use-
ful in the design of second and third generation compounds
for the treatment of TrkA.

Fig. 7. Effect of 5E5 on neurite out-
growth of PC12 cells. A, effect of 5E5
on neurite bearing cells. PC12 cells
were stimulated with 1 µM 5E5, 10
and 100 ng/ml NGF, and 1 µM 5E5
plus 10 ng/ml NGF in complete me-
dium for 4 days, with a change of me-
dium every 2 days. Cells were consid-
ered to be neurite-bearing when the
neurite length was >1.5-fold of cell
body diameter. In each instance, eight
random fields were scored for neurite
outgrowth. Data are presented as per-
centage of neurite bearing cells among
the total cells (mean ± S.D.). Asterisks
indicate statistical significance relative to 10 ng/ml NGF only
group (**, p < 0.01). B, effect of 5E5 on
neurite length. The total length of
neurites in the field was divided by
the total number of cells, giving the
average neurite length for each cell.
The treatments are shown on x-axis,
and the average neurite length (mi-
crometers) on y-axis (**, p < 0.01 ver-
sus 10 ng/ml NGF alone). C, morphol-
ogy of PC12 cell after different treat-
ments (magnification, 100×).

neuroprotective effect in the neurotrophin-withdrawal-in-
duced cell death model used for the asterriquinone. However,
we were able to demonstrate that 5E5 potentiates the effect
of low dose NGF to cause neuronal differentiation. This is
likely related to the observation that 5E5 and NGF have
additive effects on TrkA activation (Supplemental Material
5). This is particular appealing for therapeutic reasons. Neu-
rotrophins are important in many neurodegenerative dis-
orders, but they are still detectable. Therefore, a therapy that
allows these lower levels of endogenous neurotrophins to
have a neuroprotective function would avoid the side effects
of generalized neurotrophin therapy, which include thermal
hyperalgesia, weight loss, and pain sensitivity.

Both PI3-kinase and MAPK signaling downstream of TrkA
are involved in the neurotrophic effects of NGF. The PI3-
kinase signaling pathway contributes more than MAPK
pathway in neuronal survival (Kaplan and Miller, 2000;
Shimoke and Chiba, 2001; Culmsee et al., 2002; Huang and
Reichardt, 2003). The MAPK pathway, on the other hand, is
essential for NGF-induced neurogenesis (Xiao and Liu,
2003). This may explain why 5E5 was better at stimulating
differentiation than promoting survival, because 5E5 is a
potent activator of ERK but a weaker activator ofAkt. A low
dose of 5E5 (3–10 µM) activates ERK as strongly as maximal
dose of NGF (100 ng/ml), and 5E5 and NGF have additive
effects on ERK (Supplemental Material 5). However, equiv-
alent doses of 5E5 activate Akt weakly, and, more impor-
tantly, impair NGF activation ofAkt when given together
(Supplemental Material 5). This likely explains the inability
of 10 µM 5E5 to protect differentiated PC12 cell from apo-
ptosis induced by NGF and serum withdrawal. We were
unable to test higher concentrations of 5E5 in this assay due
to cytotoxicity.

In summary, by screening asterriquinone and mono-in-
dolyl-quinone libraries we were able to identify small mole-
cule activators of TrkA, the NGF receptor. These compounds
activate TrkA directly in cells and protect differentiating PC12 cells from apoptosis or promote neuronal differentiation. The QSR model that we have constructed will guide further optimization of these compounds to maximize activator activity and minimize toxicity. Although 1H5 and 5E5 themselves are still unsuitable as drug candidates, they provide lead compounds that can be put into preclinical models of neurodegeneration for proof-of-principle experiments.

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References


