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Identification and Characterization of Compounds That Potentiate NT-3-Mediated Trk Receptor Activity

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ABSTRACT

Neurotrophins are a family of secreted proteins that play an important role in the development, differentiation, and survival of neurons. Studies also suggest that aberrant neurotrophin signaling may play a role in processes underlying disease states such as schizophrenia, Alzheimer's disease, and depression. Whereas the development of agents that selectively stimulate neurotrophin signaling has proven to be difficult, compounds have been identified that potentiate neurotrophin 3 (NT-3)-mediated activation of trk A. In the present studies, we extend those initial observations to identify compounds that also potentiate NT-3-mediated activation of trk B. Compound potentiation of NT-3 was observed using several readouts of transfected and endogenous trk receptor activity, including trk receptor phosphorylation, mitogen-activated protein kinase phosphorylation, reporter assay activity (β-lactamase and luciferase), cell survival and neurite extension assays. Studies using chimeric trk receptors demonstrated that the extracellular domain is essential for compound potentiation and rule out interaction with intracellular signaling molecules as a mechanism of compound activity. Thus, the present studies demonstrate that trk B receptor activity can be potentiated by small-molecule compounds via the extracellular domain of the receptor and provide reagents for further evaluating the role of NT-3-mediated trk A and trk B activity in vivo.

Neurotrophins are a family of secreted proteins that play an important role in the development, differentiation, and survival of neurons. This family is composed of four members, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). The neurotrophins are homodimers of highly basic 120-residue polypeptides that bind and activate two classes of specific neurotrophin receptors: the trk family of receptor tyrosine kinases, and the low-affinity neurotrophin receptor p75. Neurotrophins exhibit selectivity for trk receptors because NGF selectively activates trk A; BDNF and NT-4 interact selectively with trk B; and NT-3 preferentially activates trk C, although it also binds and activates trk A and trk B (Ryden and Ibanez, 1996). Neurotrophin interaction with the trk receptors results in ligand-induced receptor homodimerization, promoting transphosphorylation of the juxtaposed trk receptor at intracellular tyrosine residues via the intrinsic receptor kinase activity. Receptor phosphorylation creates docking sites for adaptor proteins that couple the receptor to downstream signal transduction pathways, leading to a variety of biological consequences, including neuronal survival and differentiation, cytoskeletal changes, and synaptic plasticity (Middlemas et al., 1994; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001).

Upon the identification of neurotrophins as important mediators of neuronal survival and differentiation, numerous studies addressed the role of these factors and their cognate receptors in processes underlying neuronal development and function (Burstein et al., 1982; Bibel and Barde, 2000; Patapoutian and Reichardt, 2001). In addition, several studies have suggested that neurotrophin signaling is important in

ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT, neurotrophin; MAP, mitogen-activated protein; CHO, Chinese hamster ovary; L-753,000, *N*-(12-β-D-glucopyranosyl-5,7,12,13-tetrahydro-1,11-dihydroxy-5,7-dioxo-6*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazo-6-yl)formamide; K-252b, (9*S*,10*R*,12*R*) 2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*: 3′,2′,1′-*k*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid; BMS355249, 13-[6-deoxy-6-[[(2*S*)-2,5-diamino-1-oxopentyl]amino]-β-D-glucopyranosyl]-3,9-difluoro-[1]benzothieno[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6*H*,13*H*)-dione dihydrochloride; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; CRE, cAMP response element; NFAT, nuclear factor of activated T cells.

processes involved in disease states such as schizophrenia (Schramm et al., 1998; Durany and Thome, 2004; Lang et al., 2004), Alzheimer's disease (Dawbarn and Allen, 2003; Terry and Buccafusco, 2003; Lang et al., 2004), and depression (Duman et al., 1997; Siuciak et al., 1997; Shirayama et al., 2002).

Whereas our understanding of the role of neurotrophin signaling in normal and disease processes has progressed at a rapid pace, the development of therapeutic agents that directly stimulate neurotrophin signaling has proven much more challenging (Pollack and Harper, 2002). Although some progress has been made in trying to use neurotrophins or derivatives as therapeutic agents, challenges in obtaining adequate bioavailability and pharmacokinetic parameters have been difficult to overcome (Dechant and Neumann, 2002). In addition, the development of small-molecule neurotrophin mimetics has also met with numerous challenges. Although there are some reports of small molecules with neurotrophic activities or compounds that activate trk receptors directly (Maroney et al., 1995, 1997, 1999; Pollack et al., 1999; Wilkie et al., 2001; Pollack and Harper, 2002), these compounds do not seem selective for trk receptor activation. A potential alternative approach for harnessing neurotrophin signaling pathways is through the use of small molecules that potentiate neurotrophin-mediated trk receptor activation. An advantage of this approach is that the increase in neurotrophin signaling would occur only in the presence of neurotrophin, preserving the spatial and temporal relationship of receptor activity. This activity was first demonstrated with the kinase inhibitor K-252b, which potentiated NT-3-mediated trk A activation at low concentrations but then inhibited kinase activity at high concentrations (Maroney et al., 1997). Subsequent to this, a related compound, L-753,000, was identified that potentiated NT-3-mediated trk A activity without inhibiting receptor activity at high concentrations (Pollack et al., 1999). In this report, L-753,000 potentiated NT-3 functional effects in several assays, including neuronal survival, neurite outgrowth, MAP kinase activation, and trk A phosphorylation and potentiation of NT-3 binding to trk A receptors. L-753,000 had no effect on trk B and trk C function or on NT-3 binding to trk B and trk C. Although these studies did not identify a domain of the receptor required for compound activity, the evidence suggested that L-753,000 interacts directly with trk A, NT-3, or both to promote NT-3-mediated receptor activation. Thus, these studies support the concept that trk receptor activity can be modulated by small-molecule compounds.

Small-molecule compounds that are receptor-dependent potentiators of neurotrophin-mediated trk receptor activity may provide an approach for addressing the potential of neurotrophin signaling in central nervous system diseases. Whereas previous studies identified compounds that potentiated NT-3-mediated activation of trk A, these compounds had no effect on trk B or trk C activity. In an effort to build on these initial findings, the present studies were undertaken to identify compounds that potentiated neurotrophin-mediated activation of trk B and trk C. Furthermore, studies were performed to determine whether compound activity was trk receptor-dependent and to identify receptor domains required for compound potentiation.

Materials and Methods

Materials. Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). The neurotrophins NT-3, NT-4, and BDNF were purchased from Research Diagnostics (Flanders, NJ). NGF, aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO). The anti-trk antibody sc-139 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase-conjugated antiphosphotyrosine antibody 4G10, anti-MAP, and anti-phospho-MAP kinase antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The secondary antibody horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Amersham (Piscataway, NJ). The secondary antibody horseradish peroxidase-conjugated rabbit anti-sheep antibody and SuperSignal West Pico chemiluminescent reagent were purchased from Pierce Biotechnology (Rockford, IL). β-Lactamase reagents were purchased from Aurora Biosciences (San Diego, CA).

cDNA Constructs. Human trk receptor sequences that correspond to the following cDNA accession numbers were used in the generation of stable and transient cell lines and as PCR templates for trk chimera construction; trk C (k1), accession number U05012; trk B, accession number U12140; and trk A, accession number NM002529. The receptor chimeras consist of the extracellular domain of one trk receptor joined to the transmembrane and intracellular domain of another trk receptor. Extracellular/intracellular domain chimeras were generated using the gene splicing by overlap extension PCR method (Horton, 1997) and Qiagen Proofstart DNA polymerase (Qiagen, Valencia, CA). The trk B/C chimera has trk B peptides Met1 to His430 fused to trk C amino acids Phe430 to Gly825. The trk C/B chimera has trk C amino acids Met1 to Thr429 fused to trk B amino acids Leu431 to Gly822. The trk A/C chimera has trk A peptides Met1 to Ser419 fused to trk C amino acids Ile434 to Gly825. The trk C/A chimera has trk C amino acids Met1 to Ser433 fused to trk A amino acids Val420 to Gly796. All final PCR products were subcloned into the EcoRI/NotI sites of pcDNA3.0 and verified by DNA sequencing.

β-Lactamase Assay. CHO NFAT/CRE-lactamase cells (Aurora Biosciences) stably transfected with trk B or trk C were seeded in 384-well plates at 2×10^4 cells/well in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM pyruvate, 25 mM HEPES, and 2 mM glutamine. The medium was replaced with serum-free media 1 h before assay. The cells were treated with compound or DMSO for 30 min at 37°C and then with neurotrophin or buffer control for 5 h. The cells were assayed for β-lactamase activity by the addition of CCF2 substrate (Aurora) for 1 h and read in an LJL Biosystems Analyst fluorescent plate reader (LJL Biosystems, Sunnyvale, CA). Data are expressed as 460 nm (blue)/530 nm (green) ratio.

Luciferase Assay. HEK293 cells were transiently cotransfected with NFAT-luciferase reporter and trk A, trk B, and trk C receptor or receptor chimeras. At 48 h after transfection, cells were seeded into 96-well white plates at 5×10^4/well. The cells were treated with compound or DMSO and then treated with neurotrophin or media for 6 h. The cells were lysed and assayed for luciferase activity using Steadylite HTS reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) or Steady-Glo luciferase reagent (Promega, Madison, WI) and read in an LJL Biosystems Analyst fluorescent plate reader. Luminescence data output is expressed as relative light units.

Western Blot. CHO NFAT/CRE β-lactamase cells transfected with the trk B receptor or PC-12 cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM pyruvate, 25 mM HEPES, and 2 mM glutamine in 35-mm dishes. The cells were changed to serum-free media for 1 h and then treated with neurotrophin for 10 min. The cells were washed 1× with PBS (4°C) and then lysed in 100 μl/well lysis buffer (4°C; 20 mM Tris, pH 7.4, 1% Nonidet P-40, 40 mM β-glycerophosphate, 2.5 Mm MgCl, and 10 mM EDTA plus protease

inhibitor cocktail; Roche Diagnostics, Indianapolis, IN). Each well was scraped and rocked on ice for at least 15 min and then centrifuged at 10,000g for 10 min. The supernatant was removed and diluted into 4× NuPAGE sample buffer (Novex, San Diego, CA) for lysates. For immunoprecipitation samples, supernatant was diluted 5-fold in radioimmunoprecipitation assay buffer, and 20 μl of pan-trk antibody (3 μg) was added to each tube and incubated overnight at 4°C. Washed Immunopure Plus protein A agarose (30 μl; Pierce) was added to each tube. The tubes were rocked at 4°C for 30 min. The resulting bead pellet was washed 3× in radioimmunoprecipitation assay buffer and finally solubilized in 1× NuPAGE sample buffer. Samples were separated by NuPAGE 4 to 12% gels and electroblotted on polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blots were blocked with 2% casein (Roche) in Tris-buffered saline/Tween 20 (0.1%). All antibody dilutions were made in this solution. Western blots were probed with anti-phosphotyrosine antibody and anti-pan trk antibody. Western blots of lysates were probed with anti-phospho-MAP or anti-MAP antibodies. Blots were developed with SuperSignal West Pico chemiluminescent reagent (Pierce).

Cell Proliferation Assay. PC-12 cells were seeded in serum containing DMEM and 2 nM NGF and grown for a total of 7 days. Media were then exchanged for serum-free media containing compound titrated from 0.1 to 100 nM in the presence or absence NT-3 and neurotrophin controls and then grown for 72 h. Media were removed and replaced with DMEM containing 1× WST-1 cell proliferation reagent (Roche). WST-1 is a substrate for cellular dehydrogenase activity used as an indicator of cell number. Plates were incubated at 37°C for 1 h. Absorbance was measured at 440 nm wavelength. SHsy5y cells were seeded in serum containing minimum essential medium/Ham's F-12 medium and 10 mM retinoic acid. Cells were cultured for a total of 5 days and then changed into serum-free minimum essential medium/Ham's F-12 medium. Compound treatment, neurotrophin treatment, and WST-1 assay were carried out as described for PC-12 cells.

Neurite Extension. PC-12a cells were cultured in DMEM plus 10% fetal bovine serum for 7 days on collagen-coated 35-mm plates in the presence of 2 nM NGF. The media were replaced with media alone or in combination with BMS355249, NT-3, BMS355249 and NT-3, or NGF for an additional 72 h. Images were captured on a Carl Zeiss Axiovision image capture system (Carl Zeiss Inc., Thornwood, NJ). Neurite extension was quantitated by calculating the percentage of cells expressing neurites greater than two times the cell body diameter as described previously (Treanor et al., 1995; Lazarovici et al., 1998).

Results

Identification of Compounds that Potentiate Trk Receptor Activity. Based on reports demonstrating that compounds similar to the kinase inhibitor K-252b potentiate NT-3-mediated activation of trk A, we sought to identify compounds that potentiate growth factor-mediated activation of trk B and trk C. A compound structure similarity search of the Bristol-Myers Squibb Co. (Wallingford, CT) chemical library was conducted based on the structures of L-753,000 and K-252b (Fig. 1, A and B). Compounds were identified and screened for the ability to potentiate neurotrophin-mediated activation of trk B and trk C. Receptor activity was measured in stable CHO cell lines coexpressing trk receptors and a β-lactamase reporter driven by NFAT and CRE response elements. In the screen, parental, trk B-, and trk C-expressing CHO lines were treated with a single concentration of NT-3, NT-4, or BDNF in the absence and presence of test compounds. Several compounds were identified that potentiated NT-3-mediated trk B activation, exhibited no activity in the absence of neurotrophin, and did not potentiate receptor activation by NT-4 or BDNF. An example of one such compound is BMS355249 (Fig. 2, A and B). No compounds were identified that potentiated trk C activity. Therefore, based on the ability of this compound to selectively potentiate NT-3-mediated trk B activation, BMS355249 was chosen for further studies to characterize the properties of compounds that potentiate trk receptor activity.

Characterization of Trk Potentiation by BMS355249. Initial assays with BMS355249 demonstrated that the compound potentiated NT-3-mediated trk B activation but had no activity on its own (Fig. 2A). Concentration-response curves for BMS355249 in the presence of a single concentration of NT-3 demonstrated that this compound potently enhanced the effect of NT-3 at trk A (EC_{50} = 45 ± 16 nM; n = 9) and trk B (EC_{50} = 28 ± 9 nM; n = 6), but did not enhance activity at trk C (Fig. 3A). To determine whether functional trk receptors were required for compound potentiation, the effects of BMS355249 were evaluated using a mutant catalytically inactive trk B receptor (Middlemas et al., 1994; Cunningham et al., 1997). In these experiments, NT-3 exhib-
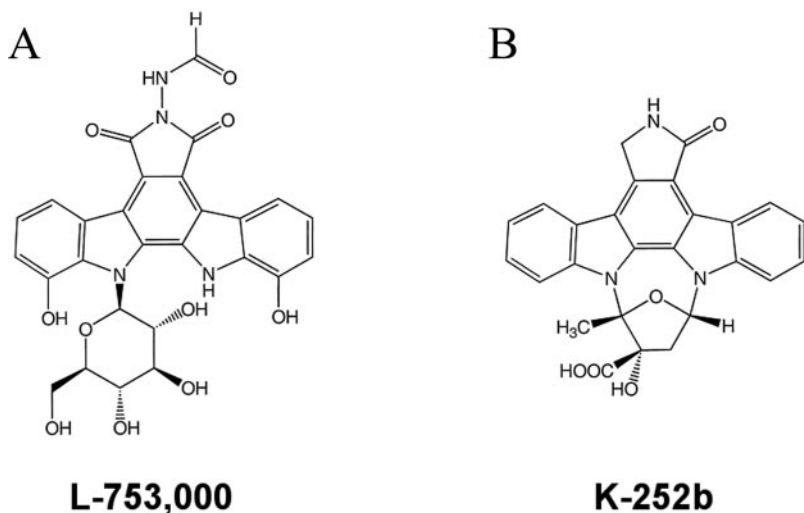

A

B

Fig. 1. Chemical structures of L-753,000 (A) and K-252b (B).

L-753,000

K-252b

ited no activity alone, and BMS355249 had no effect (Fig. 3B), demonstrating that trk receptor activity was required for compound activity. Potentiation of trk receptor activity by BMS355249 was specific for NT-3 because no potentiation of

trk A or trk B was observed in the presence of NGF and BDNF (Fig. 3, C and D), further demonstrating the selectivity of compound activity for specific trk receptors and NT-3. To understand the effects of this compound on NT-3 potency
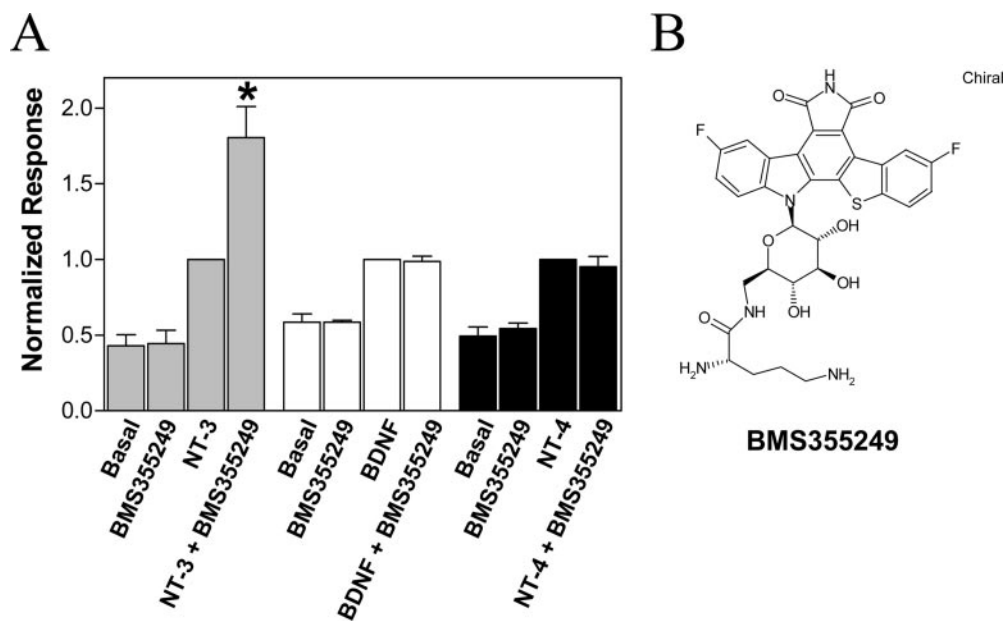
Fig. 2. Identification of trk B potentiator compounds. A, CHO/NFAT/CRE β-lactamase cells stably expressing trk B were pretreated with 10 μM compound or DMSO for 30 min and then treated with 25 ng/ml neurotrophin (NT-3, BDNF, and NT-4) and assayed for lactamase activity at 5 h after treatment. Values were normalized to the response of neurotrophin alone. Active compounds were defined as compounds that did not exhibit activity alone but potentiated the response to neurotrophin. Data were analyzed by analysis of variance using the Bonferroni post test. NT-3 alone produced a significant effect ($p < 0.01$), whereas BMS355249 had no effect alone ($p > 0.05$), and the response to NT-3 plus BMS355249 was significantly ($p < 0.01$) higher than with NT-3 alone (∗). BMS355249 did not have a significant effect on BDNF or NT-4 activity ($p > 0.05$). B, chemical structure of BMS355249.
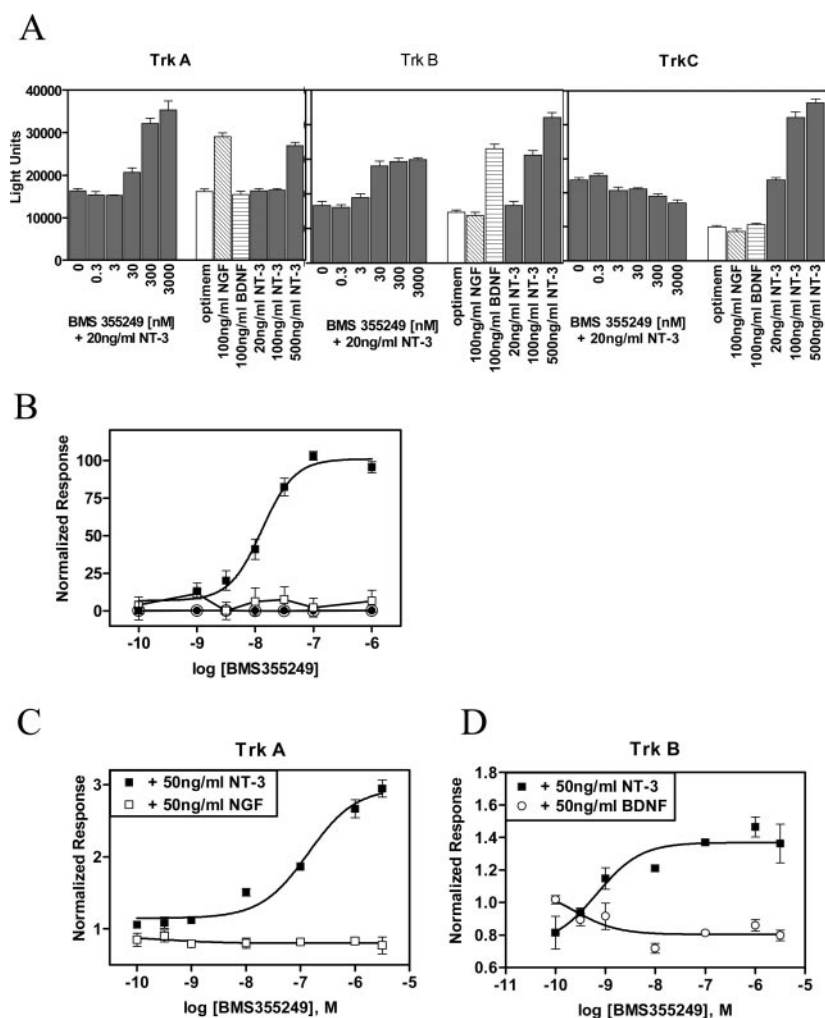

Fig. 3. BMS355249 potentiates activity at trk A and trk B not at trk C. HEK293 cells transiently transfected with either trk A, B, or C and a luciferase reporter (A, C, and D) or CHO stable cell lines (B) expressing wild-type and mutant trk B were used to measure BMS355249 potentiation of the NT-3 response. A, cells were treated with increasing concentrations of BMS355249 in the presence of 20 ng/ml NT-3 and assayed for luciferase activity as described. Control neurotrophin treatments were NGF at 100 ng/ml, BDNF at 100 ng/ml, and NT-3 at 20, 100, and 500 ng/ml. B, concentration-response curves of BMS355249 were performed in the presence (filled symbols) or absence (open symbols) of 20 ng/ml NT-3 using wild-type ($\square$, $\blacksquare$) and kinase-dead ($\bigcirc$, $\bullet$) trk B receptors and assayed for lactamase activity as described. Presented values were normalized with basal values equaling 0% activity and the NT-3 response at the wild-type receptor equaling 100%. C and D, concentration-response curves of BMS355249 were performed in the presence of 50 ng/ml NT-3 ($\blacksquare$), NGF ($\square$), and BDNF ($\bigcirc$) in cells expressing trk A receptors (C) and trk B receptors (D) and were assayed for luciferase activity as described. Presented values were normalized to the response for each neurotrophin alone. Data are the means of triplicate determinations and are representative of three separate experiments.

and efficacy, NT-3 concentration-response curves at trk B were evaluated in the presence of a single concentration of BMS355249. In these experiments, BMS355249 produced a leftward shift in the potency of NT-3 with little to no effect on maximal activity (Fig. 4), demonstrating that BMS355249 enhanced NT-3 potency. Thus, in these experiments, BMS355249 potently enhanced NT-3-mediated trk activity at trk A and trk B, increased NT-3 potency, and required the presence of functional trk receptors, indicating that the ef-

Fig. 4. BMS355249 increases the potency of NT-3 at the trk B receptor. CHO/NFAT/CRE β-lactamase cells stably expressing trk B were pre-treated with 100 nM BMS355249 (■) or DMSO (□) for 30 min and then were treated with increasing concentrations of NT-3 and assayed for lactamase activity as described under *Materials and Methods*. Data are expressed as a 460/530 nm emission ratio.

fects of BMS355249 are specific for NT-3 receptor activation and are not due to the general potentiation of trk or to tyrosine kinase activity.

Compound Potentiation Is Dependent on the Receptor Extracellular Domain. To further characterize domains of trk A and B required by compounds to potentiate activation by NT-3, we constructed receptor chimeras of trk A, B, and C. Previous reports with chimeric epidermal growth factor and platelet-derived growth factor receptors (Wilkie et al., 2001) and a chimeric insulin receptor-related receptor and trk B receptor (Kelly-Spratt et al., 2002) have demonstrated that the extracellular and intracellular domains of receptor tyrosine kinases can be used to change ligand specificity and activation of signaling pathways. Because BMS355249 demonstrated selectivity for potentiating trk A and trk B activity but not trk C, we constructed chimeric trk receptors containing the extracellular domain of trk A and B fused to the transmembrane and intracellular domain of trk C and chimeric receptors with the extracellular domain of trk C fused to the transmembrane and intracellular domains of trk A and trk B (Fig. 5A) to evaluate receptor domains of trk A and trk B involved in compound potentiation. The chimeric constructs, A/C, C/A, B/C, and C/B were evaluated in luciferase assays after transient transfection in HEK293 and CHO cells. Control experiments demonstrated the extracellular receptor domain of each chimera conferred
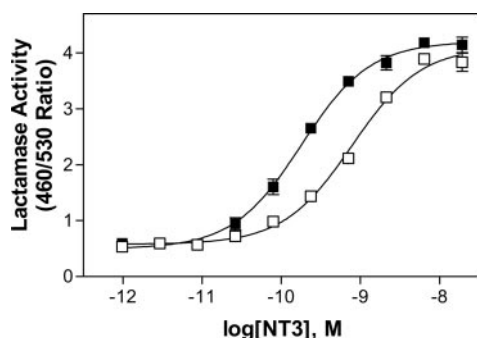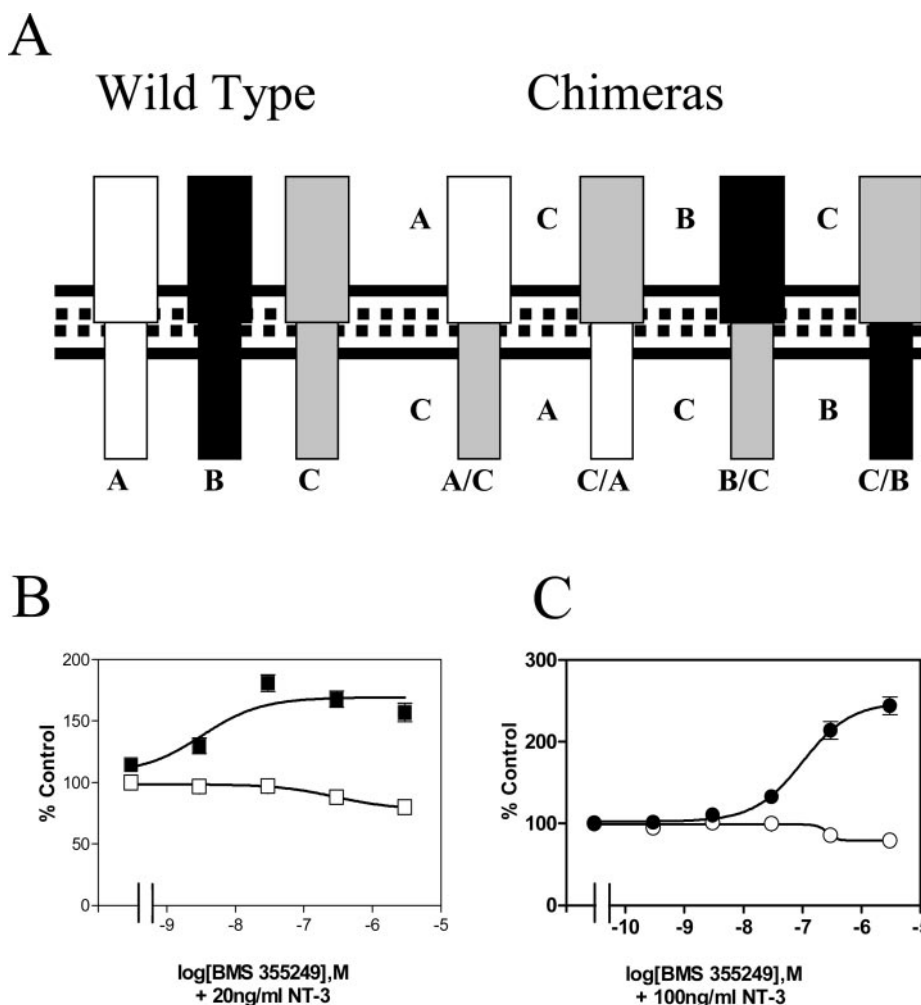

Fig. 5. Potentiation of signaling using trk receptor chimeras. Trk receptor chimera constructs were evaluated in HEK293 cells using the luciferase assay. A, schematic illustration of chimeric trk receptors as described under *Materials and Methods*. The receptor domain is indicated by the corresponding letter of the trk receptor: trk A (A), trk B (B), and trk C (C). B, trk receptor B/C (■) and C/B (□) chimeras were treated with increasing concentrations of BMS355249 in the presence of NT-3 20 ng/ml and were assayed for luciferase activity as described under *Materials and Methods*. C, trk receptor A/C (●) and C/A (○) chimeras were treated with increasing concentrations of BMS355249 in the presence of NT-3 100 ng/ml (A/C) or NT-3 20 ng/ml (C/A) and assayed for luciferase activity as described under *Materials and Methods*. Data are the means of quadruplicate determinations and are representative of at least four separate experiments.

ligand selectivity to the appropriate neurotrophin because BDNF activated the B/C but not the C/B chimera and NGF activated the A/C but not the C/A chimera (data not shown). To evaluate the ability of BMS355249 to potentiate NT-3 signaling, cells were treated with increasing concentrations of BMS355249 in the presence of a single concentration of NT-3. In these experiments, BMS355249 potentiated NT-3 activity at the A/C and B/C chimeric receptors but did not potentiate activity at the C/A and C/B receptor chimeras (Fig. 5, B and C), demonstrating that the extracellular domain of the trk A or trk B receptor is required for BMS355249 activity.

BMS355249 Potentiates Trk Receptor Phosphorylation and MAP Kinase Activation. Studies to identify and characterize compounds that potentiate trk receptor activation used reporter assays as an index of trk receptor activity. Because reporter assays are the culmination of many steps in the signaling pathway activated by trk receptors, we further characterized the effects of BMS355249 on trk receptor phosphorylation and MAP kinase activation to determine whether signaling processes more proximal to receptor activation were potentiated by the compound. Initial assays were performed on CHO cells stably expressing trk B receptors. In experiments evaluating trk receptor phosphorylation, cells were treated with neurotrophins, cell lysates were immuno-precipitated with anti-trk receptor antibodies, and immuno-precipitated eluates were evaluated by Western blot with anti-trk receptor and anti-phosphotyrosine antibodies. In control studies, BDNF, NT-3, and NT-4 increased trk receptor tyrosine phosphorylation (data not shown), demonstrating that trk receptor phosphorylation was detectable in the

Fig. 6. BMS355249 potentiates NT-3-mediated trk receptor and MAP kinase phosphorylation. A, trk receptor phosphorylation was evaluated in CHO trk B cells after treatment with neurotrophin and BMS355249. Cells were treated as indicated with neurotrophin for 10 min in the absence or presence of 100 nM BMS355249, immunoprecipitated with anti-pan trk antibodies, and immunoblotted with either anti-PAN trk or anti-phosphotyrosine antibody (4G10). Duplicate Western blots were probed with anti-trk receptor antibodies to confirm that equal amounts of trk receptor protein were immunoprecipitated. B, MAP kinase phosphorylation was evaluated in CHO cells after treatment with neurotrophin and 100 nM BMS355249. Cells were treated as indicated with neurotrophin for 10 min in the absence or presence of 100 nM BMS355249, and MAP kinase phosphorylation was evaluated by Western blot analysis with phospho-MAP kinase antibodies. Duplicate Western blots were probed with anti-MAP antibodies to confirm equal amounts of MAP kinase protein across samples.

cell line. In studies to address the effects of BMS355249 on trk receptor phosphorylation, cells were first treated with BMS355249 (300 nM) alone to determine whether the compound itself altered receptor phosphorylation. In these experiments, BMS355249 produced no change in receptor phosphorylation (Fig. 6A). In contrast, in cells treated with NT-3, the increase in trk B phosphorylation produced by NT-3 alone was further increased in the presence of BMS355249 (Fig. 6A), consistent with the effects of the compound to potentiate the trk B functional response. It is noteworthy that control Western blots using anti-trk antibodies demonstrated that the observed increase in phosphorylation was not due to changes in the amount of trk present in the immunoprecipitation (Fig. 6A).

In studies evaluating trk receptor-mediated MAP kinase activation, transfected CHO cells were treated with neurotrophin in the absence and presence of BMS355249 followed by the preparation of cell lysates and analysis by Western blot with anti-phospho MAP kinase and anti-MAP kinase antibodies. Initial control experiments demonstrated that BDNF and NT-3 increased MAP kinase phosphorylation, consistent with known properties of trk receptor signaling (data not shown) (Greene and Tischler, 1976; Burstein et al., 1982; Cowley et al., 1994; Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003). In experiments evaluating receptor potentiation, cells treated with NT-3 in the presence of BMS355249 (300 nM) yielded a larger increase in MAP kinase phosphorylation than did cells treated with NT-3 alone (Fig. 6B). In addition, cells treated with BMS355249 in the absence of NT-3 did not exhibit an increase in MAP kinase phosphorylation, indicating that the compound alone did not activate MAP kinase (Fig. 6B). Western blots using anti-MAP kinase antibodies demonstrated that equal amounts of MAP kinase were present in each treatment condition.

BMS355249 Potentiates Activation of Endogenous Trk Receptors. Studies involved in the identification and characterization of potentiator compounds used heterologous systems expressing trk receptors to evaluate the compounds. To determine whether BMS355249 potentiated NT-3-mediated activation of endogenous trk receptors, we evaluated MAP kinase activation in PC-12 cells, which endogenously express trk A receptors. In these studies, PC-12 cells treated with BMS355249 in the absence of neurotrophin yielded a minimal signal with the anti-phospho MAP kinase antibodies (Fig. 7). When cells were treated with NT-3 (100 ng/ml) in the absence of BMS355249, NT-3 produced detectable MAP kinase phosphorylation. However, cells treated with NT-3 in
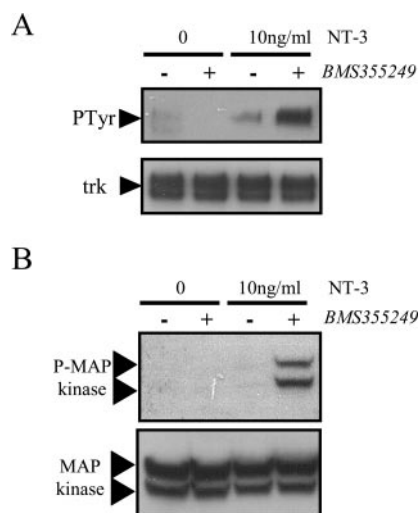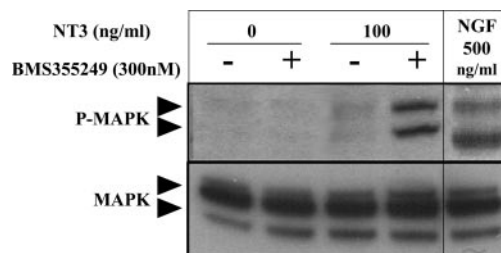

Fig. 7. BMS355249 potentiates NT-3-mediated MAP kinase phosphorylation in PC-12 cells. PC-12 cells were treated for 10 min with vehicle or NT-3 (100 ng/ml) in the presence or absence of BMS355249 (300 nM); as a control, cells were treated with NGF (500 ng/ml). Lysates were immunoblotted with anti-phospho-MAP kinase (top) and anti-MAP kinase (bottom) antibodies.

the presence of BMS355249 produced a robust increase in MAP kinase phosphorylation comparable with the NGF-mediated response (Fig. 7), revealing that BMS355249 potentiated NT-3 activity in PC-12 cells at endogenous trk receptors.

To further explore the effects of potentiating NT-3 receptor activation in cells expressing endogenous trk receptors, the effects of BMS355249 on cell survival and neurite extension were evaluated. Cell proliferation assays were established using the WST-1 cell proliferation reagent with PC-12 and SHsy5y cells, which express endogenous trk A and trk B receptors, respectively. Therefore, in PC-12 cells, NGF and serum promoted cell proliferation, whereas BDNF and serum each supported cell proliferation in SHsy5y cells (Fig. 8, A and B, bottom). NT-3 had minimal effect on cell proliferation in PC-12 cells and a small effect in SHsy5y cells (Fig. 8, A and B). In contrast, when PC-12 and SHsy5y cells were treated with NT-3 in the presence of BMS355249, cell proliferation was further enhanced compared with the effects of NT-3 in the absence of BMS355249 (Fig. 8, A and B). No effect was observed in cells treated with BMS355249 in the absence of NT-3 (Fig. 8, A and B). In both cell lines, potentiation of the NT-3 response yielded an effect on cell survival comparable with or greater than the response to either NGF (PC-12 cells) (Fig. 8A) or BDNF (SHsy5y cells) (Fig. 8B).

PC-12 cells respond to neurotrophic factors such as NGF by differentiating into neuron-like phenotypes characterized by neurite outgrowth. (Greene and Tischler, 1976; Burstein et al., 1982; Cowley et al., 1994). Other studies in PC-12 cells have demonstrated an important role for MAP kinase in NGF-dependent neuronal differentiation and neuritogenesis (Fukuda et al., 1995; Pang et al., 1995). Based on the NT-3 potentiation effects of BMS355249 on trk receptor phosphor-

ylation and MAP kinase activation, experiments were designed to investigate the relationship between NT-3 potentiation and enhancement of neurite outgrowth. In these experiments, cells were serum-starved and treated with BMS355249, NT-3, NT-3 plus BMS355249, or NGF and then quantitated for changes in neurite extension as described under *Materials and Methods* (Fig. 9). In these assays, cells exposed to vehicle, BMS355249, and NT-3 exhibited few cells exhibiting neurites (1.1 ± 0.6, 4 ± 2, and $2.9 \pm 1.6\%$, respectively) (Fig. 9, A–C). In contrast, cells treated with NT-3 in the presence of BMS355249 produced a significant ($p < 0.001$) increase in neurites ($20.3 \pm 3\%$) compared with the effect of vehicle or compound alone, comparable with that produced by NGF ($13.9 \pm 1.3\%$) (Fig. 9, D and E).

Discussion

Activation of the trk family of receptors plays an important role in processes underlying neuronal development and potentially in disease states. Small-molecule compounds that potentiate neurotrophin-mediated trk receptor activity may provide a pharmacological approach to regulate trk receptor signaling to help further understand the therapeutic potential of these receptors. In the present studies, we identified the compound BMS355249 as a selective potentiator of NT-3-mediated receptor activity. Compound activity was not dependent on cell type or assay readout, because potentiation was observed in assays using both transfected and endogenous trk receptors. In addition, compound activity was not due to nonspecific mechanisms, because BMS355249 did not have effects alone or on BDNF, NT-4, NGF, or trk C-mediated signaling. These findings support the conclusion that the present studies identified small-molecule compounds which selectively potentiated NT-3-mediated trk A and trk B receptor activity.

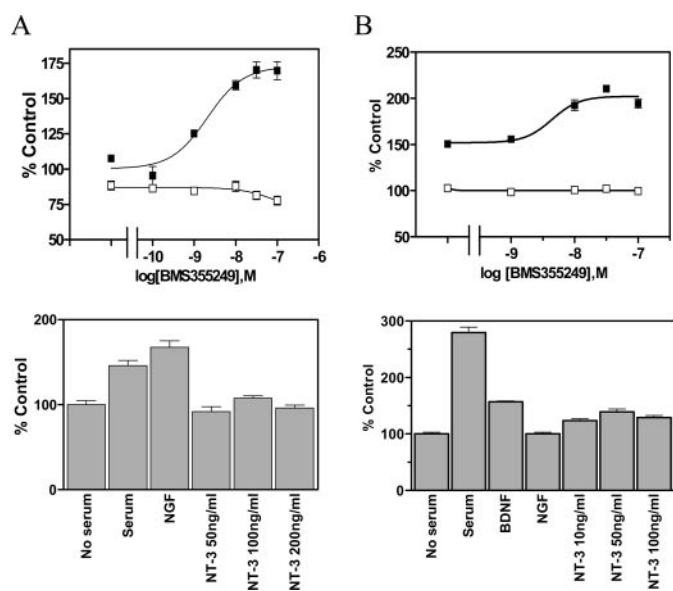Previous studies with L-753,000 and K-252b suggested

Fig. 8. BMS355249 potentiates NT-3-mediated cell survival. SHsy5y and PC-12 cells were differentiated as described under *Materials and Methods*. Cells were changed to serum-free medium with neurotrophin alone or coadministered with BMS355249 for 72 h and then assayed for survival using the WST assay as described under *Materials and Methods*. WST assay absorption values were normalized to the response of cells treated with no serum. For cell proliferation controls (bottom), cells were treated with either no serum, 10% serum, NGF, BDNF, or NT-3 as indicated. A, cell survival in PC-12 cells with BMS355249 alone (□) or in combination with NT-3 (■). B, cell survival in SHsy5y cells with BMS355249 alone (□) or in combination with NT-3 (■).
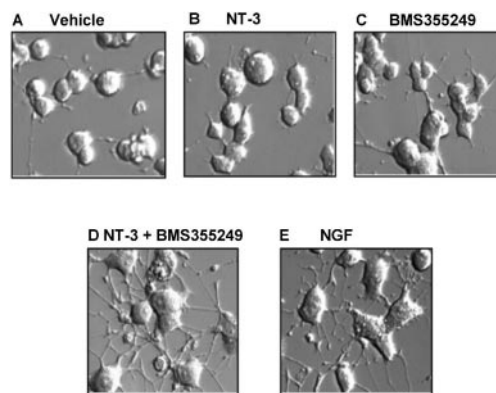
Fig. 9. BMS355249 potentiates NT-3-mediated neurite extension. PC-12 cells were grown in culture as described under *Materials and Methods* and then changed to serum-free medium, treated for 72 h as indicated, digital images were captured, and the percentage of cells exhibiting neurites was determined as described under *Materials and Methods* and is indicated below. Data are from three independent experiments, and the percentage of cells exhibiting neurites was quantitated in four to six fields per treatment containing 97 to 310 cells per field. A, vehicle, serum-free media ($1.1 \pm 0.6\%$ cells); B, 100 ng/ml NT-3 ($2.9 \pm 1.6\%$ cells); C, 100 nM BMS355249 ($4 \pm 2\%$ cells); D, 100 ng/ml NT-3 plus 100 nM BMS355249 ($20.3 \pm 3\%$ cells); E, 100 ng/ml 2.5s NGF ($13.9 \pm 1.3\%$ cells). Data were analyzed by analysis of variance using Dunnett's post-test comparing all treatments with vehicle. The responses to NT-3 and BMS355249 alone were not significant ($p > 0.05$), whereas the responses to NT-3 plus BMS355249 and NGF were significant ($p < 0.001$).

that potentiation of trk activity by these compounds was probably due to interaction with the receptor (Pollack et al., 1999). Several lines of evidence support the conclusion that BMS355249 potentiation of trk receptor activity was also receptor-dependent. First, compound activity was selective for NT-3-mediated trk activation, because compounds did not potentiate the activity of BDNF, NT-4, or NGF and had no effect in the absence of NT-3. Second, compounds were selective for trk A and B, because no potentiation of NT-3 mediated trk C activity was observed. In addition, compound activity required the presence of active trk receptors, because no activity was observed with mutant catalytically inactive trk B receptors or in the absence of trk receptors. Cumulatively, these data support the conclusion that the effects of BMS355249 are receptor-dependent. To complement these studies, we further explored the mechanism of compound activity through the use of chimeric receptors composed of the extracellular domains of trk A and trk B fused with the intracellular domain of trk C, and the extracellular domain of trk C fused with the intracellular domain of trk A and trk B. These studies demonstrated that trk receptors containing the extracellular domain of trk A and trk B and the intracellular domain of trk C were potentiated by treatment with BMS355249. In contrast, receptors containing the extracellular domain of trk C fused to the intracellular domains of trk A and B were not potentiated by the compound. Thus, these results demonstrate that the extracellular domain is essential for compound potentiation and rule out interaction with intracellular signaling molecules as a mechanism of compound activity.

Similar to previous findings, we identified compounds that potentiated NT-3- but not NGF-mediated activation of trk A. We also found that these compounds potentiated NT-3- but not BDNF-mediated activation of trk B. No compounds were identified that potentiated activation of trk A and trk B by their preferred ligands, NGF and BDNF, or trk C activation by NT-3. This result is somewhat surprising but could be related to the differences in binding site interactions between the different neurotrophins and trk receptors. For example, studies evaluating the binding of NT-3 to trk receptors demonstrated that two positively charged residues, Arg31 and His33, play an important role in NT-3 interactions with trk A and B but not trk C (Ibanez et al., 1993; Ryden and Ibanez, 1996). These results suggest that NT-3 interacts with its nonpreferred receptors in a manner distinct from its interaction with trk C. Furthermore, corresponding basic residues in NGF have a minimal effect on its interaction with trk A, indicating that trk A interactions with NT-3 are driven by different or additional determinants than trk A interactions with NGF. Thus, whereas chimeric receptor studies helped to identify the receptor extracellular domain as necessary for compound potentiation, future studies will be designed to further define the receptor and NT-3 domains required for compound potentiation to enhance our understanding of the molecular requirements for compound activity.

Whereas numerous studies have demonstrated that NT-3 can activate trk A and trk B in vitro, the physiological significance of this interaction is not entirely clear. Trk C is clearly the preferred receptor for NT-3, but there is evidence suggesting that NT-3-mediated trk A and trk B activation also occurs in vivo (Bothwell, 1995; Snider et al., 2002). For example, analysis of NT-3 and trk C receptor knockout mice

demonstrated that the NT-3 null mice exhibited a more severe neuronal phenotype than the trk C receptor knockouts, suggesting that some effects of NT-3 are mediated through receptors other than trk C (Tessarollo et al., 1997; Tessarollo, 1998). Consistent with this, Coppola et al. (2001) evaluated the effects of inserting the BDNF coding sequence in the NT-3 gene locus to activate trk B receptors in the spatial and temporal manner of NT-3. In these studies, BDNF rescued some aspects of the NT-3 knockout phenotype, suggesting that NT-3-mediated trk B activation is important during neurogenesis and that some of the functions of NT-3 may be mediated through the activation of trk B (Coppola et al., 2001). In light of the trk receptor-potentiating profile of BMS355249, this compound may be a useful tool for further evaluating the role of NT-3 activity at trk A and trk B receptors in neuronal functions.

Neurotrophin signaling is an important mediator of neuronal development and function, and numerous studies suggest that abnormal neurotrophin signaling may be involved in pathophysiological states such as depression, schizophrenia, and Alzheimer's disease. Identification of potent, selective modulators of trk receptor activity provide tools for further evaluating the role of neurotrophin signaling in these disease states. Furthermore, the demonstration that small molecules potentiate trk receptor activation by NT-3 suggests that other combinations of peptide ligands and receptor tyrosine kinases may also be amenable to potentiation by small molecules. In particular, compounds that potentiate BDNF-mediated trk B activation may be beneficial in the treatment of depression. Using the screening approach described in the present studies, future studies will be designed to evaluate this possibility.

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