Small molecule BDNF mimetics activate TrkB signaling and prevent neuronal degeneration in rodents

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Brain-derived neurotrophic factor (BDNF) activates the receptor tropomyosin-related kinase B (TrkB) with high potency and specificity, promoting neuronal survival, differentiation, and synaptic function. Correlations between altered BDNF expression and/or function and mechanism(s) underlying numerous neurodegenerative conditions, including Alzheimer disease and traumatic brain injury, suggest that TrkB agonists might have therapeutic potential. Using in silico screening with a BDNF loop–domain pharmacophore, followed by low-throughput in vitro screening in mouse fetal hippocampal neurons, we have efficiently identified small molecules with nanomolar neurotrophic activity specific to TrkB versus other Trk family members. Neurotrophic activity was dependent on TrkB and its downstream targets, although compound-induced signaling activation kinetics differed from those triggered by BDNF. A selected prototype compound demonstrated binding specificity to the extracellular domain of TrkB. In in vitro models of neurodegenerative disease, it prevented neuronal degeneration with efficacy equal to that of BDNF, and when administered in vivo, it caused hippocampal and striatal TrkB activation in mice and improved motor learning after traumatic brain injury in rats. These studies demonstrate the utility of loop modeling in drug discovery and reveal what we believe to be the first reported small molecules derived from a targeted BDNF domain that specifically activate TrkB. We propose that these compounds constitute a novel group of tools for the study of TrkB signaling and may provide leads for developing new therapeutic agents for neurodegenerative diseases.

Introduction

Nerve growth factor (NGF), brain-derived neurotrophin factor (BDNF), and neurotrophin-3 (NT-3) are members of the neurotrophin protein family and act through their cognate tropomyosin-related kinase (Trk) receptors (NGF/TrkA, BDNF/TrkB, NT-3/TrkC) and the common neurotrophin receptor p75 (p75NTR). Trks are activated by binding of mature neurotrophin dimers and multimerization (though the precise mechanisms remain unclear), leading to phosphorylation and signaling adaptor recruitment (1). Trk activation promotes neuronal survival, differentiation, and synaptic function (1–3). p75NTR signaling is complex, involving coreceptor (e.g., sortilin) interactions, proteolytic processing, and endocytosis, with coupling to both survival and apoptosis-inducing mechanisms (e.g., refs. 4–6). Engagement of p75NTR by neurotrophins also modulates Trk activity (7, 8). BDNF and TrkB are of particular therapeutic interest. Correlations between alterations in BDNF expression and/or function and mechanism(s) occurring in Alzheimer disease (9), Huntington disease (10), Parkinson disease (11), Rett syndrome (12), traumatic brain injury (TBI), (13, 14), and aging (15) point to the therapeutic potential of TrkB agonists. The findings that TrkB is important for long-term survival, differentiation, and function of newborn neurons in the adult hippocampus (16–18), and that neurogenesis plays a fundamental role in depression, suggest that discovery of TrkB ligands might open new treatment avenues for this disorder (19). Finally, modulation of TrkB signaling could have a therapeutic role in obesity or anorexia (20).

A number of properties limit the therapeutic application of BDNF. Its plasma half-life in rats is less than 1 minute, and it has poor blood brain barrier penetration (21) and poor brain intraparenchymal penetration (22). In addition, BDNF interaction with p75NTR might contribute to its ability to promote pain (23) and other undesired effects. Thus, a long-sought goal has been the development of non-peptide, small molecule ligands capable of activating TrkB signaling with high potency and specificity (24). In the present study, we applied in silico screening with a pharmacophore modeled on a BDNF loop domain likely interacting with TrkB, coupled with low-throughput in vitro neurotrophic assays to identify the first small molecule TrkB ligands mimicking a BDNF active site.

Results

Computational modeling, pharmacophore generation, and virtual screening. There was no available structure, for any neurotrophin-Trk pair, of the binding of the β-loops of interest to the receptor, and no known BDNF-derived oligopeptides with TrkB-activating activity with which to constrain the model. Therefore, we began

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Conflict of interest: F.M. Longo is a founder of PharmatrophiX, a company focused on the development of small molecule ligands for neurotrophin receptors.

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with previous studies of receptor binding and biologic activity of chimeric mutant proteins generated by successive substitution of homologous BDNF regions into an NGF backbone, which identified regions of BDNF involved in TrkB activation and Trk specificity (25, 26). Examination of those results suggested loop II subregion b (SKGQL) (Figure 1), a region sufficiently restricted to produce a small molecule model, which was associated with a substantial portion of BDNF-like activity and not apparently critical for TrkB binding, as a candidate for pharmacophore modeling. This region was modeled and a chemical feature hypothesis (shown in Figure 1A) generated, as described in Methods. Using this pharmacophore, an average of 35 conformers of each of more than 1,000,000 available compounds (including libraries from Asinex, Comgenex [now AMRI], Interbioscreen, Sigma-Aldrich [Rare Chemical Library], Timtec, and Chemstar) were screened, yielding 1,785 candidate compounds that fit with a calculated internal energy of less than 10 kcal/mol. This number was further reduced to 14 by visual inspection and other criteria, as follows: (a) since the pharmacophore used did not provide for excluded spaces, a rough (visual, qualitative) filter was applied that disfavored compounds that, in their lowest energy conformation best fitting the pharmacophore, had portions that would interfere with the interaction of pharmacophore-determined compound chemical features with a putative shallow-pocket receptor site (this criterion was the most robust in excluding compounds); (b) compounds were favored in which pharmacophore-correspondent features were connected by several rotatable bonds, providing theoretical flexibility of relative positioning; (c) compounds of lower molecular weight were favored, but those greater than 500 Da, up to 650 Da, were not automatically excluded — otherwise, Lipinski’s rule of 5 criteria was applied; and (d) compounds marginal on any of the preceding criteria, and also barely fitting the pharmacophore, could be excluded. Of these 14 compounds, 7 were obtained from commercial sources for in vitro analysis.

Identification of BDNF loop II mimetics with neurotrophic activity. Compounds were screened for neurotrophic activity using E16 hippocampal neurons under conditions in which survival is, in part, dependent upon the addition of exogenous BDNF. In preliminary assays, 5 compounds emulating the loop II region of BDNF were considered to have activity significantly above baseline as defined as a percentage of BDNF maximal efficacy. Four compounds with the highest activity (LM22A-1 to -4) were selected for further characterization. Each of these 4 compounds exhibited a distinct chemotype; LM22A-1 is a tripeptide (Pro-His-Trp), and compounds LM22A-2, LM22A-3, and LM22A-4 contain no peptide bonds (Figure 1B).

Neurotrophic activity dose-response studies with the 4 compounds demonstrated maximum levels of activity of 80%–89% of that of BDNF and EC50 values of 200–500 pM (Figure 2, A–F). TUNEL/DAPI staining confirmed the compounds’ ability to prevent neuronal death with an efficacy similar to that of BDNF (Figure 2G). Since the plateau of survival for LM22A-1 and -4 was not well established until approximately 100 nM, to ensure maximum effects, we chose 500 nM as a standard concentration for many of the subsequent mechanistic studies.

In beginning to examine the mechanism of action of these compounds, we considered the possibility that they might act as secretagogues, increasing BDNF levels. Anti-BDNF antibody treatment of hippocampal cultures resulted in a decrease in BDNF neurotrophic activity but had no effect on the activity of the 4 test compounds (Figure 2H), supporting the view that these compounds are not BDNF secretagogues.

LM22A compounds interact with and act through TrkB. The hypothesis that the LM22A compounds act through TrkB was examined through multiple approaches. The finding that compounds had maximal activities at 80%–89% of that of BDNF suggested that if they functioned as a ligand, or by otherwise co-opting TrkB or its downstream signaling pathways, they might inhibit BDNF function by approximately 10%–20%. In contrast, additive activity would indicate a parallel mechanism. In hippocampal cultures, addition of 500 nM LM22A-4 with 20 ng/ml (~0.7 nM) BDNF, concentrations of each which induce a maximal response, produced no additive effect on survival (Figure 2I). Similar results were obtained with LM22A-3

Figure 1
Pharmacophore and LM22A compound structures. (A) Inset: Variable loop regions of a human BDNF monomer extracted from the crystallographically determined structure of a BDNF-NT3 heterodimer (56). The larger image shows the loop IIb (sequence SKGQL) pharmacophore hypothesis, as described in the text. Red, hydrogen bond donor; green, hydrogen bond acceptor. (B) Structures of the 4 LM22A compounds chosen for these studies. Compounds are abbreviated as #1–#4 in subsequent figures.
If these compounds work through activation of TrkB, they should be blocked by inhibition of receptor activation. Treatment of hippocampal neuron cultures with the Trk inhibitor K252a led to a reduction in the neurotrophic activity of BDNF and all 4 test compounds. K252a has been reported to also block mixed-lineage kinases, leading to inhibition of JNK activation, and to activate AKT and ERK through the Src pathway (27), which lead to improved survival; however, we saw no improvement in survival with K252a alone in our cellular systems. In addition, as an alternative approach, we examined the effects of an antibody directed against the extracellular domain (ECD) of TrkB (anti-TrkB ECD) that is known to inhibit BDNF function (28). This resulted in a reduction in cell survival (Figure 3B) and increased numbers of TUNEL-positive cells in the presence of BDNF, LM22A-1, and LM22A-4. LM22A compounds specifically activate TrkB. To examine the effects of LM22A compounds on different Trk family members, we determined Trk phosphorylation in NIH-3T3 cells expressing single Trks. In 3T3-TrkB cultures, addition of BDNF or LM22A compounds resulted in TrkB activation, while NGF was inactive (Figure 3D). However, in 3T3-TrkA and 3T3-TrkC cultures, the neurotrophins produced the expected results, while the LM22A compounds induced no Trk activation (Figure 3D). Thus, the LM22A compounds selectively activate TrkB.

LM22A-4 was selected as a prototype compound to further examine Trk interactions, as it appeared the simplest and most flexible structurally, and most amenable to chemical modifica-
Figure 3
LM22A compounds function through TrkB. (A) Survival analysis of hippocampal neurons treated with BDNF (0.7 nM) or LM22A compounds (500 nM) with or without the Trk inhibitor K252a (200 nM). For BDNF, n = 37 wells derived from 7 experiments; for LM22A-1, -2, and -3, n = 17–21 wells derived from 6 experiments; and for LM22A-4, n = 28–57 wells derived from 4 experiments were assessed. (B) Survival analysis of hippocampal neurons treated with CM, BDNF (0.7 nM), or LM22A compounds (500 nM) with or without TrkB ECD antibody. For each condition, n = 37–42 wells derived from 5 experiments were assessed. (C) TUNEL/DAPI analysis of E16 hippocampal neurons treated with CM, BDNF (0.7 nM), or LM22A compounds (500 nM) with or without TrkB ECD antibody. For each condition, n = 17–31 fields derived from 7 experiments were assessed. (D) LM22A compounds or neurotrophic factors were incubated for 60 minutes with NIH-3T3 cells stably expressing specific Trk receptors. Western blot analysis (anti–pan-phospho-Trk antibody) demonstrated that LM22A compounds activated TrkB (upper panels) but not TrkA (middle panels) or TrkC (lower panels), while all 3 Trks were activated by their cognate ligands. Activation patterns of 3 additional independent assays were identical. (E–H) Trk- and p75NTR-specific NIH-3T3 cells were incubated in serum-free medium in the presence of the designated ligands for 72–96 hours, and survival was measured using the EnzyLight assay. *P < 0.05, **P < 0.01, ***P < 0.001.

LM22A-4 binds specifically to TrkB. LM22A-4 was incubated with chimeric TrkB-Fc and ephrin-A5-Fc (as a negative control) and found to bind to the TrkB ECD-Fc but not ephrin-A5ECD-Fc protein, and addition of BDNF blocked the binding of LM22A-4 to TrkB ECD-Fc (Figure 4A). To further examine LM22A-4-TrkB interactions, the displacement of BDNF from TrkB ECD-Fc-Cy3B by the compound was determined by measuring changes in fluorescence anisotropy of the receptor with increasing LM22A-4; this was compared to the effects of the compound on labeled TrkB in the presence of a nonbinding neurotrophin, NGF (Figure 4B). Measured anisotropy (r), proportional to TrkB bound, fell from approximately 0.31 to approximately 0.17 between 0 and 75 nM LM22A-4, with an IC50 of 47 nM or less. In the presence of NGF, without LM22A-4, there was no increase in fluorescence anisotropy above baseline, and the further addition of the compound had no effect. To further address specificity of receptor binding and binding in a cellular context, we performed competition studies with 3T3 cells expressing each of the neurotrophin receptors. LM22A-4 inhibited binding of BDNF to TrkB-expressing cells but had no detectable effect on cells expressing TrkA, TrkC, or p75NTR (Figure 4, C–F). In addition, a screen of 57 receptors performed by Cerep Inc. detected no significant binding (Supplemental Figure 1). These results are consistent with LM22A-4 acting selectively through TrkB.
encompassing the onset and plateau of its promotion of survival (Figure 5A). Of note, TrkB activation was not significantly increased at an LM22A-4 concentration of 0.1 nM, though ERK and AKT were detectably activated at this level. In examining kinetics, BDNF caused substantial TrkB Y490 phosphorylation within 10 minutes of application to hippocampal neurons in culture, and these levels were maintained for up to 240 minutes (Figure 5B). In contrast, the response to 500 nM LM22A-1, -2, and -3 was nominal for up to 30 minutes, with increased but relatively low levels of activation at later time points. LM22A-4 showed intermediate levels of TrkB activation over a time course similar to that of BDNF. Interestingly, treatment of hippocampal neurons with BDNF and LM22A compounds led to robust increases in AKT and ERK phosphorylation within 30 minutes, with profiles similar to BDNF thereafter (Figure 5, C and D). This early discrepancy between TrkB phosphorylation and AKT/ERK activation is reminiscent of that seen in the LM22A-4 concentration series (Figure 5A) and suggested the possibilities that in these cells, downstream mechanisms are highly sensitive to small changes in TrkB phosphorylation state or, less likely, that the compounds stimulate coordinated signaling to AKT and ERK through another site on TrkB or act through some other mechanism. To test whether LM22A compound signaling is dependent on the presence and activation of TrkB, we examined the effects Trk inhibition on compound signaling in cultured hippocampal neurons and in cells expressing one of TrkA, TrkB, or TrkC. We found that anti-TrkB antibodies substantially inhibited TrkB, AKT, and ERK activation induced by LM22A-3 and -4 in hippocampal neurons (Supplemental Figure 3, A–D). In addition, LM22A-1 to -4 each induced AKT and ERK phosphorylation in TrkB-expressing fibroblasts but not in TrkA- or TrkC-expressing cells (Supplemental Figure 4, A and B). Finally, we found that inhibiting TrkB phosphorylation in 3T3-TrkB cells with K252a was associated with inhibition of compound-induced AKT and ERK activation (Supplemental Figure 5, A–D). Of note, K252a alone had no effect on AKT or ERK activation in these cells. These findings suggest that signaling induced by the LM22A compounds specifically requires TrkB and is associated with TrkB activation/phosphorylation. The question of the differences in TrkB activation kinetics between BDNF and the compounds remains for further study.

To further establish the pathways involved in LM22A activity, we investigated whether the compound’s neurotrophic activity was dependent on AKT and ERK activation. LY294002, a specific inhibitor of PI3K (an upstream activator of AKT), and the MAPK kinase (an upstream activator of ERK) inhibitor PD98059 reduced the neurotrophic activity of BDNF and the LM22A compounds (Figure 5E). Together, these findings suggest that LM22A compounds induce survival of hippocampal neurons in culture through interactions with TrkB and downstream activation of PI3K/AKT and MAPK/ERK pathways.

**LM22A-4 activates TrkB, AKT, and ERK in vivo.** To determine whether a TrkB small molecule, non-peptide ligand would be capable of activating TrkB in the adult mammalian brain, LM22A-4 was selected for in vivo studies. Preliminary pharmacokinetic studies in mice indicated that LM22A-4 exhibited very low blood brain barrier penetration (brain concentrations for 10 mg/kg LM22A-4 given i.p. for 1 hour, 62 ± 7 nM; 3 hours, below detectable limit; by liquid chromatography/tandem mass spectrometry [LC-MS/MS], Absorption Systems); therefore, the compound was applied to adult mice by intranasal administration. After once-daily administration of vehicle or LM22A-4 for 7 days, brains were harvested 2.5 hours after the last dose, and hippocampal and striatal tissue were assessed for Trk, AKT, and ERK activation. LM22A-4 induced an increase in activation of Trk, AKT, and ERK in hippocampus and striatum (Figure 6, A–C). Given that a small portion of the cell population within each sampled tissue expressed TrkB, the apparent increases in TrkB, AKT, and ERK activation in whole tissue extracts point to particularly robust signaling effects in these brain regions.

**LM22A-4 matches BDNF efficacy in preventing neuronal death in vitro and in vivo models of neurodegenerative diseases.** Since the LM22A compounds upregulated fundamental survival/stress resistance mech-

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**Figure 4**
LM22A-4 binds selectively to TrkB. (A) LM22A-4 was incubated with ephrin A5-Fc receptor as a negative control or with TrkB<sup>ECOD</sup>-Fc in the absence or presence of BDNF (600 pmol). After rinsing of receptor complexes and subsequent ligand elution, LC/MS-MS demonstrated readily detectable LM22A-4 binding to TrkB<sup>ECOD</sup>-Fc that was blocked by BDNF. Values were derived from 3 independent binding studies. (B) BDNF (100 nM, black circles) or NGF (100 nM, white squares) was incubated with TrkB<sup>ECOD</sup>-Fc-Cy3B (100 nM) and increasing concentrations of LM22A-4; fluorescence anisotropy (<<>) was measured for 6–9 times in single samples at each concentration. (C–F) NIH-3T3 cells expressing TrkB, TrkA, TrkC, or p75<sup>NTR</sup> as indicated were incubated with increasing concentrations of the indicated neurotrophin in the absence (white squares, dashed line) or presence (black circles, solid line) of 100 nM LM22A-4. Symbols represent average fluorescent signal after background (i.e., binding to cells lacking expressed receptors) subtraction, as described in Methods. n = 14–18 wells from 7–9 experiments at each concentration. Significant rightward shifting of the binding curve by the compound was observed only with TrkB expressing cells.

*P < 0.05, **P < 0.01, ***P < 0.001.
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anisms, we hypothesized that they would mitigate neuronal death across a variety of mechanisms and disease models and examined the effects of LM22A-4 in models of Alzheimer, Huntington, and Parkinson diseases. Accumulation of toxic species of Aβ is likely to play a critical role in the pathogenesis of Alzheimer disease, and the neurodegenerative effect of Aβ in neuronal cultures serves as an in vitro model (29). Previous studies have demonstrated that BDNF protects neurons from Aβ-induced neuronal death (30). In 6–7 days in vitro (DIV) hippocampal neurons treated with oligomeric Aβ, BDNF and LM22A-4 each led to a similar substantial reduction in Aβ-induced death, and this protective activity was inhibited by the Trk inhibitor K252a, consistent with a Trk-dependent mechanism (Figure 7A). Application of MPP⁺ (metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]), a mitochondrial toxin, to SH-SYSY human tumor line cells (31) is a widely used Parkinson disease model. Here, BDNF and LM22A-4 blocked MPP⁺-induced cell death to a similar extent, and this protective activity was inhibited by K252a (Figure 7B). Finally, a model for Huntington disease involves the administration of the excitotoxin quinolinic acid (QA), an N-methyl-D-aspartate receptor agonist (32, 33). In preliminary studies, QA caused increasing cell death in cultures of striatal neurons, from 1.25 to 50 mM; 7.5 mM was selected in order to ensure a clear toxic effect. BDNF and LM22A-4 led to a similar substantial reduction in QA-induced death, and this protective activity was again inhibited by K252a (Figure 7C). Together, these studies indicate the potential broad application of TrkB agonists to neurodegenerative processes.

LM22A-4 improves motor learning after TBI. Motor learning and plasticity have been associated with TrkB and/or BDNF expression in the cortex (34, 35) and cerebellum (34), and BDNF has

Figure 5

Comparison of BDNF’s and LM22A compound’s signaling activation kinetics and dependence of survival on downstream signaling. (A) Left: Representative Western blot showing activation of Trk, AKT, and ERK in cultured E16 hippocampal neurons incubated with LM22A-4 at the indicated concentrations for 60 minutes. Right: Western blot analyses were quantitated for Trk, AKT, and ERK activation (ratio of p-Trk to total TrkB; p-AKT to total AKT; and p-ERK to total ERK signal), n = 15–18 Western analyses from 4–9 independent protein preparations. (B–D) Cultured E16 hippocampal neurons were incubated with BDNF (0.7 nM) or LM22A compounds (500 nM) for the indicated times, and quantitative analysis for Trk, AKT, and ERK activation was performed as in A. n = 8–11 Western blot analyses from 4–5 independent protein preparations. For each Western blot, the activation ratio induced by BDNF at the 10-minute time point was normalized to 1.0 and the other values adjusted accordingly. (E) E16 hippocampal neurons were incubated with BDNF (0.7 nM) or LM22A compounds (500 nM) for 48 hours in the presence or absence of the PI3K inhibitor LY294002 (LY, 10 μM) or the MAPK kinase inhibitor PD98059 (PD, 50 μM). For BDNF, n = 33–37 wells derived from 7 experiments were assessed. For LM22A-1, -2, and -3, n = 17–21 wells derived from 6 experiments were analyzed. For LM22A-4, n = 28–57 wells derived from 4 experiments were assessed. *P < 0.05, **P < 0.01, ***P < 0.001.
been reported to improve motor recovery and increase measures of neuronal remodeling after cortical ischemia (36, 37) and cerebellar pedunculotomy (38). Though BDNF infusion has been reported to have no effect on spatial learning after TBI (39, 40), motor learning was not assessed in those studies. We therefore examined the effects of LM22A-4 on changes in rotarod performance over repeated trials, a measure of motor learning (41), after TBI. Rats subjected to parietal controlled cortical impact injury showed a trend toward decreased rotarod performance relative to sham-operated animals at 1 week after TBI, and this was similar to sham-operated controls, whereas animals subjected to TBI and treated with vehicle showed no improvements (Figure 8). With repetition at 2 and 3 weeks after injury, LM22A-4–treated animals showed significant improvements in performance, indistinguishable from sham-operated animals at 21 days after injury, there was no difference between the treated and vehicle groups (Supplemental Figure 6). This suggests that LM22A-4 may reverse deficits in motor task learning caused by TBI, though it has no effect on gross total injury.

**Discussion**

These studies describe what we believe to be the first reported small molecules mimicking a specific neurotrophin domain, capable of functioning as ligands to selectively and potently activate the TrkB receptor.

We initially theorized that using loop subdomain pharmacophore modeling and virtual screening, the a priori likelihood of identifying compounds functioning through TrkB would be substantially greater than that for compounds identified in conventional high-throughput screening for neurotrophic activity (which would identify compounds functioning through any mechanism). As in our previous studies on p75NTR (42), we found that this method provides a manageable group of compounds for biologic testing, with a high rate of recovery (5 activated of 7 tested) of the desired activity relative to that which would be obtained using high-throughput screening (~1%, refs. 43, 44), and similar to that of other virtual screens beginning with more structural information (45). Multiple lines of experimental evidence suggest that these small molecules function as TrkB ligands, including: specific activation of TrkB and two primary downstream signaling intermediates; inhibition of activity by the kinase inhibitor K252a and a monoclonal antibody directed to the ECD of TrkB; blocking of compound binding to the ECD of TrkB by BDNF; compound effects on TrkB-expressing, but not TrkA- or TrkC-expressing, NIH-3T3 cells; and inhibition of BDNF binding to TrkB, with no inhibition of neurotrophin binding to TrkA, TrkC, or p75NTR, in addition to a negative Cerep screen. While the present studies failed to detect interaction of LM22A-4 with p75NTR or prevention of cell death through p75NTR, future studies will address whether such ligands might nevertheless affect its complex intracellular adaptor/signaling milieu. The lack of effect of BDNF antibodies, compound inhibition of BDNF maximal activity, and LM22A-4 induction of greater neurite outgrowth than maximal BDNF suggest that the LM22A compounds do not act as BDNF secretagogues. Thus, we suggest that the identification, within this small group of tested compounds, of chemically diverse compounds sharing the common mechanism of TrkB activation supports the validity of the model employed in these studies.

Recent studies show that small molecules may bind with relatively high affinity to protein surfaces through “hot spots,” small groups of residues that contribute significant energy to the protein-protein interaction of interest. Small molecule binding can disrupt protein-protein interactions and inhibit the functions they mediate (46–50). Moreover, the small molecule may induce conformational changes in the binding domain that differ from those associated with binding of the natural ligand (48). Given the findings of small molecule–mediated TrkB agonism presented here, and our previous findings with p75NTR ligands (42), we suggest that hot-spot binding and induction of conformational changes in the receptor may allow these small molecules to act as activating ligands, though there may be differences from the natural ligand with respect to the coupling and kinetics of the induced signaling. Further structural studies of compound-receptor complexes will be needed to confirm this hypothesis.

Of note, other natural ligands of TrkB may also induce signaling quantitatively and qualitatively different from that induced by BDNF. Mutation of the SHC-binding site of TrkB in mice caused loss of NT-4–dependent, but not BDNF-dependent, neurons in vivo and decreased survival of sensory neurons in culture and ERK activation in response to NT-4 relative to BDNF (51). In another study, c-fos promoter activity in cortical neurons was found to be more responsive to exogenous NT-4 than BDNF (52). Such differential activation of downstream signaling by ligands may reflect and/or play an active role in partial agonism and may therefore be significant in the effects of LM22A compounds on endogenous BDNF actions. Moreover, the effects of differential activation by small molecule ligands may vary with respect to other native TrkB ligands (NT-4, NT-3) and lead to unexpected inhibition or synergy. Further studies comparing signaling activation kinetics and pathways between the compounds and protein ligands, including coexposure, will be needed to evaluate these possibilities.

**Figure 6**

LM22A-4 activates Trk, AKT, and ERK signaling in vivo. LM22A-4 or saline control was administered intranasally (0.22 mg/kg/d) to adult mice for 7 days. Protein extracts of hippocampus and striatum were assessed by Western blot analysis for (A) Trk, (B) AKT, or (C) ERK phosphorylation. *n = 11 mice for each assay. *P < 0.05.
Additionally, it would be reasonable to expect that the methodology presented here would also yield pure BDNF/TrkB antagonists, though we have not specifically assayed for these as of yet. Unfortunately, the small numbers of compounds and lack of available close structural analogs make detailed structure-activity relationship analysis infeasible at this point; this will be addressed in future studies employing larger libraries and/or synthesis of directed analogs.

The lack of rotational symmetry of LM22A-1 and -3 and the small size of the compounds suggest that they are functionally monovalent (i.e., do not interact with more than one receptor monomer). This apparent monovalency raises interesting questions regarding mechanisms of TrkB activation by the compounds. Previous studies have characterized synthetic peptides modeled on loop regions of BDNF, with the goal of using active peptides to derive active non-peptide small molecules that might function as BDNF mimetics (24, 53, 54). In each of these studies, peptides with neurotrophic activity were found; however, the ability to activate TrkB was either not described (24, 53) or not detected (54). Hence whether such peptides can function as ligands that activate TrkB remains to be determined. Unlike TrkA and TrkC, for which there is evidence that “monovalent” cyclic peptidomimetics can exhibit small degrees of agonist activity (55, 56), for TrkB only “dimeric” peptides have as yet been found to demonstrate trophic activity. This finding is consistent with the classical model in which the BDNF dimer is thought to induce dimerization leading to activation (autophosphorylation) of TrkB. If the compounds act in a monomeric fashion, one possible mechanism by which this activation might occur is that the compound binds to a TrkB monomer, inducing a conformational change that promotes dimerization. Also, if, as has been proposed for TrkA, the unliganded receptor monomer is in equilibrium with dimeric or higher multimeric states (57), then the compounds might stabilize and/or promote phosphorylation of complexes, without inducing dimerization. It is unlikely that each of the 4 compounds characterized here is capable of forming high-affinity, bivalent dimers in solution, which then function as dimeric ligands. Co-crystallization and other future kinetic and structural studies will address the questions of how the mechanisms of activation and binding of the small molecules to TrkB compare with those of BDNF.

The ability of the prototype compound LM22A-4 to trigger activation of TrkB and its downstream signaling intermediates in the hippocampus and striatum is consistent with the observed effects on motor learning after TBI, though the anatomic substrates and mechanisms of that effect will require further investigation.

These findings, along with the compound’s ability to improve cell survival in several in vitro neurodegenerative disease models, open new opportunities for the further development of derivatives that may have therapeutic effects in a large number of acute and chronic neurological and neuropsychiatric disorders.
that small molecule TrkB ligands could have the BDNF-like effects of promoting neurogenesis and synaptic function suggests further novel therapeutic applications of such compounds. The finding that BDNF interaction with p75NTR promotes mechanisms underlying pain (23) raises the interesting possibility that small molecule targeting of p75NTR interactions might reduce the likelihood of pain as a side effect occurring in BDNF-based therapeutics. In addition, the partial agonist profile of the compounds described here may support their use under circumstances where excessive TrkB signaling is undesirable.

The methods and compounds described here provide a basis for drug discovery in other receptor systems and for designing and characterizing novel non-peptide TrkB ligands optimized for blood brain barrier penetration and other pharmacological features.

**Methods**

**Materials.** 5-Oxo-l-prolyl-l-histidyl-l-tryptophan methyl ester (LM22A-1, catalog BAS 0380372), 2-[2,7-bis[(2-hydroxyethyl)amino]sulfonyl]-9H-fluoren-9-ylidine]-hydrazinecarboxamide (LM22A-2, catalog BAS 0461519), and N′-[4-[2-[[5-amino-4-cyano-1-(2-hydroxyethyl)-1H-pyrazol-3-yl]-2-cyanoethenyl][phenyl]-acetamide (LM22A-3, catalog BAS 0548227) were purchased from Asinex Corp., with purity of more than 90% determined by NMR spectroscopy. N,N′,N″-tris-(2-hydroxyethyl)-1,3,5-benzentriacarboxamide (LM22A-4, catalog CHS 0233913) was purchased from ChemBridge Corp., with purity of more than 95% determined by NMR and LC-MS. The molecular weights and formulae of these 4 compounds were further confirmed in our laboratory by high-resolution mass spectrometry. Other chemicals were purchased from Sigma-Aldrich unless otherwise stated. Other sources include the following: LY294002, K252a, and PD98059, Calbiochem; mouse monoclonal anti-phospho-ERK 

**Assays employing in vitro models for Alzheimer, Huntington, and Parkinson disease were based on previously published protocols and are described below.**

**NIH-3T3 cell cultures and survival assays.** Mouse NIH-3T3 cells expressing TrkA (NIH-3T3-TrkA) or p75NTR (NIH-3T3-p75NTR) were provided by William Mobley, Stanford University. NIH-3T3 cells expressing TrkB (NIH-3T3-TrkB) or TrkC (NIH-3T3-TrkC) were provided by David Kaplan, Montreal Neurological Institute, Montreal, Quebec, Canada. Cells were propagated in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 200–400 μg/mlycin (for Trk-expressing cells) or 400 μg/ml hygromycin (for p75NTR-expressing cells). Cells were seeded into 24-well plates (30,000 cells/well) and cultured in medium consisting of 50% PBS and 50% DMEM without supplements. After exposure to growth factors or compounds for 72–96 hours, cells were suspended in 50 μl PBS and transferred to 96-well plates, and survival was measured using the EnzoLight Cytotoxicity Assay Kit (BioAssay Systems).

**LM22A-4 binding assays.** For direct binding measurements, protein G agarose beads (10 μl) were washed twice with PBS binding buffer (pH 8.0) and incubated with 300 pmol TrkBECID-Fc or ephrin-A5ECID-Fc at room temperature.
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Protein extraction and Western blot analysis

For assays of Trk, AKT, and ERK activation, hippocampal neurons derived from E16 mice were cultured in poly-L-lysine-coated 96-well plates (Fisher Scientific). After reaching 85%–95% confluency, data were derived by subtracting background signal, which was determined by the signal obtained from the wells containing NIH-3T3 cells lacking neurotrophin receptors incubated with a maximal level of neurotrophin. Data were normalized to the signal representing maximal binding of each neurotrophin to cells expressing its corresponding receptor. LM22A-4 was submitted to Cerep Inc. for receptor binding screening using standard protein coupling protocols recommended by the manufacturer. Samples containing varying concentrations of BDNF or PBS, with or without K252a (200 nM) and 30 minutes later, were added to the wells. After incubation for 48 hours, the number of surviving cells in each of 8 wells per condition was determined by MTT morphology criteria.

LM22A-4–induced signaling in vitro

Adult male C57BL/6J mice (2–4 months of age) were randomized into vehicle (normal saline [NS]) and treatment (LM22A-4 dissolved in NS) groups. Mice were given 10–15 μl vehicle or a 0.5-μg/ml solution of LM22A-4 into the right naris 3–5 times at 2- to 4-minute intervals (providing a dose of 0.22 mg/kg body weight), once daily for 1 week. Hippocampal and striatal brain regions were harvested 2.5 hours after the last dose and processed for Western blot analysis.

TBIs, LM22A-4 treatment, and rotated performance

Male Sprague-Dawley rats (8 weeks old) were anesthetized with 80 mg/kg ketamine/10 mg/kg xylazine i.p. A rectal thermistor and heating pad were used to maintain rectal temperature at 37°C throughout the procedure and recovery. The skull was exposed by midline incision and reflection of the scalp and temporalis muscle, and a 4-mm craniotomy was placed over the left parietal cortex midway between the lambda and bregma sutures. The incisions were then sutured, and the animals were allowed to recover. Sham-operated animals received a craniotomy but no injury. Injured animals received 3.4 μg LM22A-4 in a gel containing Carbopol 934P (0.7%), hydroxypropyl methylcellulose (1.3%), triethanolamine (2.5%), and methylparaben (0.05%) to enhance retention and delivery (64) or vehicle alone, with 15 μl delivered to the left nare immediately after injury, then daily for 14 days. Motor testing was performed on an accelerating rotarod (increasing 1 rpm/5 s; SDI Rotor-Rod, San Diego Instruments Inc.) with time to fall recorded. Animals received training of 2 trials on the 2 consecutive days prior to TBI and then were tested with 2 trials weekly thereafter by an observer blinded to treatment. To evaluate compound effects on lesion size, male Sprague-Dawley rats injured and treated as above were terminally perfused with phosphate-buffered saline containing 4% paraformaldehyde (PFA) 21 days after injury, and their brains were postfixed in 4% PFA overnight, followed by soaking in 30% sucrose for 48 hours. Serial sections encompassing the injured area were cut at 40-μm intervals using a Leica CM1850 microtome. Sections were Nissl stained and every fifth section evaluated in a blinded fashion for lesion size, using Adobe Photoshop pixel counting.

Statistics

Statistical analyses applied the Student’s t test, ANOVA with Dunnett’s or Tukey-Kramer correction, or repeated-measures ANOVA, with pairwise multiple comparisons using the Holm-Sidak method. For all graphs, mean ± SEM is shown. α = 0.05 for all tests.

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