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Novel Small Molecule Activators of the Trk Family of Receptor Tyrosine Kinases

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Abstract

The Tropomyosin-related kinase (Trk) receptors are a subset of the receptor tyrosine kinase family with an important functionality in the regulation of neurotrophic signaling in the peripheral and central nervous system. As the receptors are able to mediate neuronal survival by associating with their respective neurotrophin ligands, many studies have focused on the therapeutic potential of generating small-molecule mimetic compounds that elicit agonistic effects similar to those of the natural protein ligands. To this end, various structure-based studies have led to the generation of bivalent peptide-based agonists and antibodies that selectively initiate Trk receptor signaling; however, these compounds do not possess the ideal characteristics of a potential drug. Additionally, the reliance of structure-based data to generate the compound libraries, limits the potential identification of novel chemical structures with desirable activity. Therefore, subsequent investigations utilized a cell-based apoptotic screen to facilitate the analysis of large, diverse chemical libraries of small molecules and quickly identify compounds with Trk-dependent antiapoptotic activity. Herein, we describe the Trk agonists that have been identified by this screening methodology and summarize their *in vitro* and *in vivo* neurotrophic activity as well as their efficacy in various neurological disease models, implicating their future utility as therapeutic compounds.

Keywords

TrkB agonist; BDNF; synthetic derivatives; antidepressant; neurogenesis

Introduction

Receptor tyrosine kinases (RTKs) are a class of enzyme-linked transmembrane glycoproteins that play a pivotal role in cellular signaling. This family of cell surface receptors are essential for the conveyance of the pleiotropic effects of growth factors, differentiation factors and hormones, which are secreted in response to external stimuli to bind a specific receptor and initiate intracellular signaling cascades (1). The importance of RTKs in the maintenance of cellular homeostasis is exemplified by their necessity for proper embryonic development and proper adult tissue functionality (2). In the human genome, there are 58 members of the RTK family, which can be divided into subclasses according to

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their structural similarities (1). In general, the RTKs possess a similar topology, consisting of a glycosylated, extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain, which catalyzes the transfer of γ-phosphate from ATP to tyrosine residues (2). Most of the receptors are composed of a single polypeptide chain and are thought to be monomeric in the absence of ligand, however, the insulin receptor subfamily exist as heterotetramers, consisting of two, dimeric polypeptide chains connected by disulfide linkages (2). Although the various receptors have similar intracellular domains, which possess catalytic activity and sites for protein-protein interactions, their extracellular domains differ greatly, possibly to confer ligand-specificity as a means of regulating receptor signaling. The diverse extracellular portions of the receptors contain distinct sequences of folding motifs, such as immunoglobin-like domains, cysteine-rich domains, leucine-rich domains and fibronectin type 3-like domains.

To facilitate intracellular signal transduction, receptor catalytic activity must be stimulated by an associating ligand. The general method of receptor activation occurs upon ligand binding to an extracellular portion of the receptor and inducing its noncovalent dimerization or a conformational change in heterotetrameric receptors, which triggers autophosphorylation of tyrosine residues within the activation loop of the receptor's tyrosine kinase domain (1). Many ligands exist as symmetric or asymmetric dimers, connected by a disulfide bond, to provoke the association of a second receptor monomer upon binding. It is unclear whether receptor dimerization induces dimer formation between the intracellular domains of the receptor, as well as its extracellular domains, or if the cytoplasmic portions of the receptors are merely brought within close proximity of one another to facilitate trans-autophosphorylation by one tyrosine kinase domain acting as the substrate for the other and vice versa (2). Nonetheless, phosphorylation, within the activation loop, stimulates receptor catalytic activity and the formation of docking sites for proteins containing phosphotyrosine-recognition motifs, such as a Src homology 2 (SH2) domain or a phosphotyrosine-binding (PTB) domain (3). Subsequently, interactions between docking proteins leads to the upregulation of a major signaling pathway, such as mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLC-γ), and phosphatidylinositol-3 kinase (PI3K) (4).

In the current review, we summarize the current published data regarding Trk receptor small molecular agonists. We focus on the screening strategy, *in vitro* biochemical validation and *in vivo* biological and pharmacological actions of these compounds. In addition, we also briefly summarize the monoclonal antibody as Trk receptor agonists and small molecular Trk receptor agonists discovered in other laboratories. With the data published at this moment, 7,8-dihydroxyflavone attracts the most attention since its publication and mimics the physiological and therapeutic actions of BDNF in various neurological disease animal models. Thus, this small molecule and its synthetic derivatives provide not only a powerful tool to dissect the biological and physiological functions of BDNF/TrkB signaling but also useful pharmacological agents for treating a variety of neurological diseases.

Trk Receptors

One subfamily of the RTKs is the tropomyosin-related kinase (Trk) receptor family, which is composed of three members, TrkA, TrkB and TrkC. The Trk receptors are a major class of neurotrophin receptors, as they promote cell survival and inhibit apoptosis in the specific populations of CNS neurons. Trk receptor signaling is activated in response to neurotrophin binding; each neurtrophin has high affinity for a particular receptor, Nerve Growth Factor (NGF) binds to TrkA, Brain-Derived Neurotrophic Factor (BDNF) and NT-4/5 bind to TrkB and NT-3 binds TrkC (4, 5). Rarely, this ligand specificity can be changed when the sequence of the receptor's extracellular domain is altered, as is observed with various splice variants; the extracellular portion of the receptors is also subject to varying amounts of post-

translational glycosylation (6, 7). As described above, the three Trk receptors exist as monomers and their dimerization is induced by the binding of their respective dimeric neurotrophins. Specifically, BDNF associates with the extracellular domain of TrkB by interacting with the third leucine-rich motif, the cysteine cluster-2 domain and the immunoglobin-2 domain, thereby stimulating receptor dimerization; TrkA and TrkC are bound and activated in a similar manner (8). Upon BDNF binding, TrkB receptor is autophosphorylated in the activation loop, of its cytoplasmic tyrosine kinase domain, at Tyr702, 706 and 707 (TrkA-Tyr670, 674 and 675), which enhances subsequent phosphorylation at two additional residues, which will act as docking sites for downstream effectors. Phosphorylation of TrkB-Tyr516 (or TrkA-Tyr490) induces an interaction between the receptor and the PTB domain of Shc, which initiates Ras-MAPK and PI3K signaling pathways and subsequently, activates Akt1/PKB (6). Similarly, phosphorylation of TrkB-Tyr817 (or TrkATyr785) facilitates association between the receptor and PLCγ1, which stimulates the release of Ca^{2+} and upregulates PKCδ (6).

Alternatively, activation of the Trk receptors has been observed, in the absence of neurotrophins, to be mediated by the G-protein-coupled receptor (GPCR) (9). Although the underlying mechanism is incompletely understood, the GPCR ligands, adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP) were able to trigger the phosphorylation of Trk receptor adapter proteins, Shc and PLCγ (9, 10). Additionally, the GPCR ligands were able to induce neuronal survival, via the PI3K-Akt pathway, analogously to NGF. Immunofluorescence experiments suggest that Trk activation is mediated by the GPCR ligands in an intracellular compartment, in a manner that partially involves the Golgi apparatus (9). Regardless of the mechanism of activation, proliferative processes within the cell can be stimulated by Trk signal transduction, fulfilling an essential function for the development and maturation of the nervous system.

Trk Receptors as therapeutic targets

The neurotrophic effects on neuronal survival elicited by the interaction between neurotrophins and their respective receptors have prompted investigations to focus on the therapeutic potential of these proteins in neurodegenerative diseases. In particular, identifying mimetic compounds that interact with the Trk receptors has been the focus of many studies since administration of exogenous neurotrophins has proven ineffective. The family of neurotrophic factors are polypeptides that are secreted to promote neurite outgrowth, neuronal differentiation and survival and their effects are mediated through their selective interactions with the members of the Trk receptor family (4).

The signaling upregulated by NGF is able to inhibit apoptosis and promote cellular differentiation, thus the neurotrophin has been shown to reverse basal forebrain cholinergic atrophy, reduce cognitive decline, stimulate cholinergic fiber growth in humans with mild Alzheimer's disease and ameliorate peripheral diabetic neuropathies (11, 12). Additionally, NGF has been proposed to have utility in the attenuation of neuronal damage and neuroectoderm-derived tumors (13). Similarly, BDNF imparts its neurotrophic effects on various neuronal populations, which have implicated the neurotrophin in several neurodegenerative diseases, such as peripheral sensory neuropathies, amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (14–17). However, supplementation of exogenous NGF and BDNF has not proven to be efficacious. Observations that NGF infusion can decrease cholinergic neuronal atrophy and enhance spatial memory retention in rats and that intracranial NGF administration can increase acetylcholine levels in the parietal cortex and hippocampus suggest that NGF may have therapeutic potential for the prevention of age-related neurodegeneration and the onset of neurodegenerative diseases (18, 19). However, there are many challenges that hinder the efficacy of neuronal growth factors,

including their bioavailability and stability. The neurotrophins are polypeptides, hence their size precludes them from crossing the blood-brain barrier and their intracranial administration produces undesirable side-effects, such as infection and changes in intracranial pressure caused by surgery (20, 21). They are also subject to proteolysis, which rapidly decreases their effective concentration, thus necessitating the design or identification of neurotrophin-mimetics (22). Initial studies focused on the use of Trk receptor structural information for the design and synthesis of agonistic peptidomimetic compounds (23).

There is a plethora of evidence that supports the use of peptide-mimetics to induce receptor activation in a manner similar to that of the parent protein agonist, such as bicyclic peptide agonists of the erythropoietin receptor and the thrombopoietin receptor, as well as monocyclic dimeric agonists of N-cadherins (24–26). These peptide-based agonists were found to associate with their respective receptors similarly to the full-length protein agonists and induce receptor dimerization. Similarly, x-ray crystal structures of the neurotrophins, NGF, BDNF, NT-3, and NT4/5, have aided the design and synthesis of peptide-based mimetics for the TrkB receptor. The neurotrophins exist as homodimers, in which each monomer contains ~120 residues arranged in seven β-strands, connected by three hair-pin loops exposed to solvent (17). The solvent exposed loops of BDNF have been found to mediate its biological activity; interestingly, replacement of the residues in the solvent-exposed loop of NGF with the corresponding residues of BDNF enables NGF to bind the TrkB receptor and induce it homodimerization (27). These observations led to the design and synthesis of a monocyclic, monomeric peptide, composed of the same 10 amino acid sequence of BDNF loop-2 and the peptide was reported to act as a TrkB antagonist (28). Subsequent bicyclic and tricyclic dimeric peptides, connected by disulfide linkages, composed of the same BDNF loop-2 sequence were found to act as a TrkB agonists and were able to promote survival of embryonic chick sensory neurons *in vitro* (17). Although an *in vivo* analysis of the compounds has not been reported, it is highly unlikely that these compounds could potentially serve as effective therapeutic agents, because of the instability of their peptide backbone. In a similar structure-based study, a small library of peptidomimetic compounds, based on the structure of the NT-3 beta-turns, was constructed and biological studies were used to identify a selective TrkC antagonist, 2CI (29). Subsequently, the NT-3-based peptide library was used to generate bivalent-fluorescent compounds, which upon screening, yielded peptidomimetics able to selectively induce TrkC-dependent neuritogenic differentiation or cell survival (30).

An alternate methodology, incorporating the concept of developing bivalent compounds to activate the neurotrophin receptors, utilizes polyclonal and monoclonal antibodies to generate agonistic characteristics. As a means of investigating the function of the low-affinity NGF receptor (LNGFR), polyclonal antibodies were prepared against the extracellular domain of rat LNGFR and used to block NGF binding to the receptor (31). The notion of using antibodies as receptor agonists was extended by producing antisera to the rat TrkA receptor, and demonstrating that the anti-receptor antibody was able to induce autophosphorylation of the receptor and the survival and outgrowth of sympathetic neurons (32). These observations are similar to those of a monoclonal antibody, specific for the insulin-like growth factor-1 receptor, which was able to stimulate receptor kinase activity and DNA synthesis (33). Similarly, monoclonal antibodies, targeted to human TrkA and TrkB receptors, have been generated and found to activate receptor signaling, and promote neuronal survival and neurite outgrowth similarly to their native ligands (34, 35). In addition to their agonistic activity, these Trk-specific antibodies represent useful tools to enhance the study of Trk signaling. Interestingly, the TrkA-specific monoclonal antibody, 5C3, is not only an NGF-mimetic, but it has also been used as a diagnostic tool in TrkA-positive neuroblastoma imaging, suggesting potential utility of these antibodies as diagnostics (36).

In an effort to generate compounds with optimal pharmacological properties, Longo and colleagues utilized computational modeling to generate a library of compounds based on the structure of BDNF loop-2 subregion b (SKGQL); after virtually screening the candidate compounds, 7 were obtained for *in vitro* analysis (37). One of the compounds, LM22A-4, lacking any peptide bonds, was found to selectively associate with the TrkB receptor and induce its autophosphorylation as well as the phosphorylation of its downstream signaling proteins, Akt and Erk (37). The agonist was also able to inhibit neuronal death and restore motor learning in *in vitro* neurodegenerative disease models. Additionally, their studies identified the first small molecule TrkB agonist exhibited low blood-brain barrier penetration and activated TrkB and its downstream targets in mice (37). Although these structure-based investigations have yielded Trk agonists with structural similarity to their respective full-length protein ligands, the ability for these compounds to be diversified and optimized is limited. Also, the compilation of candidate compound libraries requires the cumbersome synthesis of multiple, complex molecules, derived from the same parent neurotrophin ligand. The elucidation of novel small-molecule Trk receptor agonists has recently been facilitated by the use of a cell-based apoptotic screen, which exploits the receptor's cell survival functionality to identify compounds with Trk-dependent agonistic activity from a diverse library. This approach not only negates the need of constructing a compound library and enables unbiased identification of unique molecular structures, but it also offers a method to rapidly assess the Trk-dependence of the compound's promotion of cell survival.

Cell-based screening to identify Trk receptor agonists

The Ye lab has utilized a novel approach to elucidate small-molecule Trk receptor activators by employing a cell-based apoptotic assay, which focuses on the desired receptor pro-survival functionality to identify agonists (38). Initially, the principle behind the assay was to identify compounds that exert neurotrophic activity, analogous to NGF, in murine basal forebrain T17 cells, which are SN56 cells that have been stably transfected with TrkA; following compound incubation with the cells, staurosporine is added to initiate apoptosis and finally a cell-permeable, caspase-3-activated fluorescent peptide, $MRDEVD)_2$ is introduced. Cells that have been incubated with compounds lacking neurotrophic activity undergo apoptosis and active caspase-3 cleaves the $MRDEVD)_2$ peptide, producing a red fluorescent signal (39). As one could imagine, this screen could easily be adapted to identify agonists or antagonists of any of the neurotrophic receptors. Initially, screening was performed with 2,000 compounds from the Spectrum Collection Library and multiple hits were found to exhibit TrkA-mediated neurotrophic effects by protecting T17 cells, but not SN56 cells, from apoptosis; the identified TrkA and TrkB agonists are described below. This counter-screening strategy ensures the positive exerts their pro-survival actions in a Trk receptor dependent manner, either through directly activating the receptor or Trk receptor-mediated downstream effectors.

Gambogic Amide is a TrkA agonist

Four of the novel neurotrophic compounds are derivatives of a natural product, gambogic acid (GA) and the major active ingredient in gamboge, a resin excreted by the *Garcinia hanburryi* tree in Southeastern Asia. Gambogic acid, as well as three of its derivatives, gambogic amide, dimethyl-gambogic acid, and dihydro-gambogic acid, was found to prevent apoptosis in T17 cells more strongly than NGF. The activity of the dimethyl-gambogic acid and acetyl iso-gambogic acid derivatives was not selective for TrkA, as they were also found to protect TrkB and TrkCexpressing cells from apoptosis. Interestingly, one of the derivative compounds, decahydro-gambogic acid was found to increase apoptosis in cells expressing TrkA and TrkB, suggesting this compound could possess anti-cancer

activity. Although NGF interacts with the extracellular immunoglobulin-like domain of TrkA, truncation mutants identified the cytoplasmic juxtamembrane domain as the region of the receptor that interacts with GA (38).

The neuroprotective effects of the derivatives were determined using primary hippocampal neurons, which expresses TrkA receptor and are known to be protected by NGF; interestingly, gambogic amide exhibited a more profound protective activity than NGF, in response to insulting stimuli glutamate and oxygen-glucose deprivation- induced apoptosis (40, 41). Neuroexcitotoxicity was also blocked by gambogic amide in mice, following induction of caspasedependent and caspase-independent apoptosis by kainic acid or middle cerebral artery occlusion (MCAO)-induced ischemia (42). In subsequent analyses, gambogic amide was found to be the only derivative that is able to activate TrkA, by selectively inducing the receptor's autophosphorylation at Y490, Y751 and Y794 and inducing its dimerization, to a greater extent than NGF. Additionally, gambogic amide was found to elicit mitogenic effects in T17 cells and primary hippocampal neurons, because of its ability to induce the phosphorylation of two prominent proteins in PI3K and MAPK signaling, Akt and Erk1/2. Furthermore, gambogic amide was found to exhibit an NGF-like effect on neurite outgrowth of PC12 cells. These studies indicate that gambogic amide may represent a clinical treatment for neurodegenerative diseases and stroke.

Amitriptyline is a TrkA and TrkB agonist

In addition to gambogic acid derivatives, several tricyclic antidepressant compounds were able to selectively prevent apoptosis in T17 cells (43). Of these compounds, only amitriptyline was able to decrease apoptosis in primary hippocampal neurons, similarly to NGF and gambogic amide, in response to glutamate or oxygen-glucose deprivation-induced neuroexcitotoxicity. Immunofluorescent staining and immunoblot analysis provide evidence that amitriptyline mediates its effects by triggering the autophosphorylation of TrkA at Y751 and Y794, but not Y490, and the upregulation of downstream signaling targets, Erk1/2 and Akt *in vitro* and in mouse brain *in vivo*. Similarly, amitriptyline was also able to mediate the autophosphorylation of TrkB *in vitro* and *in vivo* at Y817. Radioactive *in vitro* binding experiments demonstrate that amitriptyline associates with the first Leucine-Rich Motif of the extracellular domain of TrkA, and that the compound binds the NGF receptor with approximately 5-fold higher affinity than TrkB. Interestingly, amitriptyline not only induces the homodimerization of TrkA and TrkB, but it also triggers heterodimerization of TrkA-TrkB, both *in vitro* and in mouse brain. The neurotrophic compound is also able to mimic one of the most notable actions of NGF, by inducing neurite outgrowth in PC12 cells and preventing kainic acid-induced neuronal apoptosis in mouse brain. Finally, the antidepressant actions of amitriptyline were assessed in mice and TrkA signaling was not found to be required for the therapeutic action of the compound, suggesting that the compound mediates its effects through its interactions with the TrkB receptor. This explanation is highly probable, as antidepressants are typically found to require TrkB signaling to exert their effects and amitriptyline has previously been reported to enhance BDNF, not NGF, in the serum of depressed patients (44, 45).

7,8-dihydroxyflavone is a TrkB agonist

The cell-based apoptotic screen can easily be modified to facilitate the identification of small-molecule agonists of other neurotrophic receptors by simply changing the cell-line used in the assay. To identify compounds able to activate TrkB signaling, T48 cells, which are SN56 cells that have been stably transfected with TrkB, were utilized in place of T17 cells and screened against a 2,000 compound Spectrum Collection Library. The initial screening results contained multiple flavone derivatives; therefore, these compounds were

subjected to additional analyses, which revealed the derivative, 7,8-dihydroxyflavone, to have potent, neuroprotective effects against apoptosis, in response to glutamate treatment or oxygen-glucose deprivation (46). 7,8-dihydroxyflavone was also the only flavone derivative to elicit TrkB autophosphorylation as well as phosphorylation of the receptor's downstream signaling targets, Erk1/2 and Akt. Similarly to BDNF, 7,8-dihydroxyflavone binds to the TrkB extracellular domain, with a K_d = 320 nM, and induces its homodimerization; however, the compound is unable to induce heterodimerization with either TrkA or TrkC. 7,8-dihydroxyflavone appears to have similar neuroprotective effects as BDNF. Neuroexcitotoxicity was initiated by kainic acid treatment or middle cerebral artery occlusion (MCAO) in TrkB F616A knockin mice, which exhibit TrkB-null phenotype when treated with 1NMPP1. In both models, as well as glutamate-treated cortical neurons, 7,8-dihydroxyflavone protected neurons from apoptosis in a TrkB-dependent manner and structure-activity relationship studies show that the 7-position hydroxyl group is critical for its agonistic effect.

Dysregulation of the important neurotrophic activity mediated by the interaction between BDNF and TrkB is observed in numerous neurological disorders, suggesting the utility for receptor agonists in their treatment. This agonistic compound appears to have a great deal of promise as a therapeutic agent, as it has been found to exhibit a great deal of efficacy in multiple mammalian disease models. In mice, treated with a dopaminergic toxicant that causes Parkinson's Diseaselike symptoms, MPTP, 7,8-dihydroxyflavone, similarly to BDNF, is able to attenuate the induced neurotoxic effects, which was evaluated by the preservation of tyrosine hydroxylase expression and the reduction of activated caspase-3 (46). In a mouse model of an advanced stage of Alzheimer's disease, another neurodegenerative disease, 7,8-dihydroxyflavone was able to rescue hippocampus-dependent memory deficits commonly associated with the disease (47). Systemic treatment with the small molecule agonist was able to enhance levels of active, phosphorylated TrkB, restoring the deficient TrkB-BDNF signaling. A similar phenomenon was observed upon the administration of 7,8-dihydroxyflavone following the exposure of rats to severe stress, which provoked long-lasting impairment of hippocampus-dependent memory (48). 7,8-dihydroxyflavone was able to restore the long-term memory of stressed rats; suggesting that TrkB-BDNF signaling has an integral role in spatial memory and that the agonistic compound may have efficacy in patients suffering from post-traumatic stress disorder (48). The small-molecule TrkB agonist is also able to attenuate the symptoms of an autistic disorder known as Rett syndrome, by improving life span, body mass, running distance, breathing abnormalities and hippocampal neuronal nuclei size (49). The effects of chronic antidepressant treatment are known to involve enhanced BDNF expression and subsequent activation of the TrkB receptor; similarly, acute administration of 7,8-dihydroxyflavone has been observed to initiate neurogenesis, emotional learning and antidepressant effects in mice (50–52). Although the compound was found to be orally available, an independent study found that intracerebroventricular administration of 7,8-dihydroxyflavone prevents the development of a depressive phenotype (51, 53). Another example of the parallel effects between BDNF and 7,8-dihydroxyflavone is the ability of the flavonoid to enhance neuromuscular transmission in the diaphragm muscle of mice; analogously, BDNF significantly enhances neuromuscular transmission in rats (54, 55). All in all, there is a significant amount of evidence supporting the therapeutic potential of 7,8-dihydroxyflavone in various neurological disorders involving TrkB signaling.

In an effort to identify the importance of the 7,8-dihydroxyflavone substituent groups, a structure-activity relationship study was performed by generating a library of flavonoid derivative compounds and measuring their neurotrophic activity (51). Upon incubation with primary rat cortical neurons, the agonistic effect of the compounds was assessed by their ability to induce TrkB autophosphorylation, as compared to BDNF. Interestingly, the 7,8-

dihydroxy catechol group on the A-ring was found to be required for the compound's agonistic activity (51). However, 7,3'-dihydroxyflavone and 7,8,3'-trihydroxyflavone were able to elicit more robust TrkB phosphorylation than the parent compound; thus, a 3'-hydroxy group on the B-ring of the compound can enhance its agonistic activity, whereas the highly hydroxylated 3,5,7,8,3',4'-hexahydroxyflavone antagonizes TrkB activation. In a quantitative approach, activation of Akt by the flavone derivatives was monitored by ELISA analysis of the primary neurons and found to coincide with the immunoblotting results that hydroxylation of the B-ring can regulate the stimulatory effects of 7,8-dihydroxyflavone, as that 2'-and 3'-hydroxylation enhance agonistic activity and 4'-hydroxylation diminishes agonistic activity. Additionally, derivatives containing 6,7-dihydroxy groups are able to induce Akt activation, suggesting that modification at these positions confers partial activity to the compound (51).

Deoxygedunin is a TrkB agonist

In addition to the flavones identified by the cell-based apoptotic screen with TrkB-expressing T48 cells, a number of gedunin derivatives were identified as well (56). This class of natural products, indigenous to India, has a tetranortriterpenoid structure and antimalarial, insecticidal and anticancer activities (57–59). The ability of deoxygedunin to prevent apoptosis, selectively in T48 cells and in a dose-dependent manner in response to oxygen-glucose deprivation in hippocampal neurons, suggests that this derivative also has neurotrophic activity. Immunofluorescent staining and immunoblotting offer additional evidence that deoxygedunin activates TrkB in primary neurons, by triggering its autophosphorylation at Y817. Similarly to BDNF, the compound is able to elicit TrkB signaling, as evidenced by the receptor's autophosphorylation and the phosphorylation of Erk1/2 and Akt in cortical neurons and in the brains of mice, suggesting that deoxygedunin is able to cross the blood-brain barrier. *In vitro* binding experiments demonstrate that deoxygedunin selectively associates with the extracellular domain of TrkB and induces its dimerization more strongly than BDNF. To demonstrate that dexoygedunin acts through TrkB receptor, we employed TrkB knockout and TrkB F616A mutant knockin neurons. Deoxygedunin substantially blocks glutamate-induced neuronal apoptosis in wild-type but not TrkB −/− neurons, indicating that it exerts its neuroprotection in a TrkB-dependent manner; whereas TrkB F616A mice, which portray a TrkB-null phenotype upon treatment with 1NMPP1 TrkB inhibitor, were used to demonstrate the TrkB-dependent neuroprotective effects of deoxygedunin in response to kainic acid treatment *in vivo*. Furthermore, BDNF conditional knockout mice, in which BDNF gene deletion is limited to the cortex, were used to provide evidence that the compound activates TrkB independently of the cognate ligand BDNF.

Upon total depletion of BDNF, as observed in $BDNF^{-/-}$ mice, severe deficiencies in coordination and balance are observed as a result of aberrant degeneration of several sensory ganglia, including the vestibular ganglion (60). As expected, deoxygedunin-treated $BDNF^{-/-}$ mice exhibit approximately 5-fold more vestibular ganglia than vehicle-treated mice, suggesting that the compound protects the vestibular ganglia from degeneration; similar observations were made with 7,8-dihydroxyflavone. Since BDNF is also known to play a critical role in mediating the therapeutic effects of antidepressants, a forced swim test was used to determine that deoxygedunin and 7,8-dihydroxyflavone have a similar effect in mice and the absence of their therapeutic effect when TrkB signaling is blocked by 1NMPP1, suggests that the antidepressant activity of the agonists is TrkB-dependent. To further examine the agonistic effects of the compound on TrkBmediated processes, mice were treated with deoxygedunin and observed to have an enhanced acquisition of the fear memory (61, 62). ^{3}H-deoxygedunin-treated mice revealed that the compound accumulates primarily in the brain, where it remains as much as 4 hours after administration, suggesting

that the compound has a favorable half-life within the body. Although deoxygedunin is orally available, its water solubility is relatively low; hence derivatives are being synthesized to improve the characteristics of the compound. Structure-activity relationship studies have found that the D-conformation of the epoxy ring is essential to observe the agonistic effects of the compound. Nonetheless, the striking similarities between BDNF and deoxygedunin, as well as 7,8-dihydroxyflavone, both *in vitro* and *in vivo* provide strong evidence of the therapeutic potential of these molecules and their utility in dissecting the molecular mechanisms of BDNF/TrkB signaling.

Future implications

Thus far, this cell-based apoptotic screen has yielded multiple small molecular agonists of the neurotrophic TrkA and TrkB receptors. The simplicity of the assay allows a wide variety of compounds, with novel structures, to be screened in a relatively short amount of time. By replacing the cell-line used to perform the screen, agonists and antagonists of any of the neurotrophic receptors could potentially be identified. The compounds that have been identified thus far are able to effectively mimic the activities of NGF and BDNF, by inducing receptor autophosphorylation, dimerization and the upregulation of secondary signaling molecules. 7,8-dihydroxyflavone and deoxygedunin are also able to potentiate antidepressant effects, as well as sensory and learning processes that are typically governed by TrkB signaling. Hence, second generation compounds, possessing higher binding affinity and solubility than the parent compounds, will have great potential as therapeutic agents for the treatment of neurodegenerative diseases.

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Highlights

- The neurotrophic Trk receptor family and their drug target potential are reviewed.
- Supplementation of endogenous ligand is an ineffective therapeutic.
- Various approaches used to generate ligand mimetics are discussed.
- Characteristics and therapeutic efficacy of agonistic molecules are summarized.