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REVIEW ARTICLE

Low-molecular mimetics of nerve growth factor and brain-derived neurotrophic factor: Design and pharmacological properties

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Abstract

To overcome the limitations of the clinical use of neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), scientists have been trying to create their low-molecular-weight mimetics having improved pharmacokinetic properties and lacking side effects of full-sized proteins since the 90s of the last century. The efforts of various research groups have led to the production of peptide and nonpeptide mimetics, being agonists or modulators of the corresponding Trk or p75 receptors that reproduced the therapeutic effects of full-sized proteins. This review discusses different strategies and approaches to the design of such compounds. The relationship between the structure of the mimetics obtained and their action mechanisms and pharmacological properties are analyzed. Special attention is paid to the dipeptide mimetics of individual NGF and BDNF loops having different patterns of activation of Trk receptors signal transduction pathways, phosphoinositide 3-kinase/protein kinase B and mitogen-activated protein kinase/extracellular signal-regulated kinase, which allowed to evaluate the contribution of each pathway to different pharmacological effects. In conclusion, data on

therapeutically promising compounds being at different stages of preclinical and clinical studies are summarized.

KEYWORDS
BDNF, dipeptide, low-molecular mimetics, neurotrophins, NGF

1 | INTRODUCTION

The neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) play a crucial role both in the nervous system formation in development and in the maintenance of normal functioning and recovery after damage in an adult organism.

NGF and BDNF perform many biological functions in neurons and a number of other cells including the regulation of proliferation, differentiation, migration, cell metabolism, synaptic plasticity, the cell cycle, support of the phenotypic stability, as well as neuroplasticity support.[1–5]

Mature neurotrophins are symmetrical homodimers. Each protomer, consisting of 118–119 amino acids, contains seven β-strands connected by three externally exposed β-turn loops (1, 2, and 4, residues 28–36, 43–49, and 91–98, respectively) and also exposed loop 3, consisting of three consecutive reverse turns (residues 59–75; Figure 1). In addition, both C- and N-terminals and a β-strand fragment (residues 79–89) are exposed to the solvent. All of these exposed sites may be accessible for interaction with neurotrophin receptors for geometrical reasons.

The neurotrophins implement their main functions through the interaction with specific high-affinity neurotrophin receptors (Trk). The neurotrophin binding to the receptor leads to the dimerization of the latter, which in turn causes phosphorylation of several tyrosine residues in the cytoplasmic domain, that results in the formation of signal

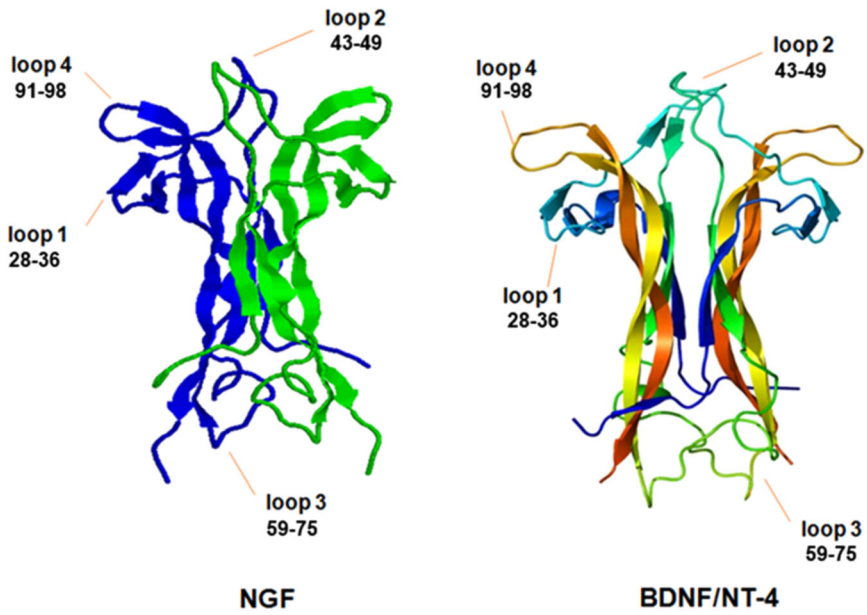


FIGURE 1 The structures of NGF homodimer (PDB ID: 1bet) and BDNF/NT-4 heterodimer (PDB ID: 1b8m). BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor [Color figure can be viewed at wileyonlinelibrary.com]

and adapter proteins binding sites and activation of three main signal transduction pathways—phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and phospholipase Cγ (PLCγ).[6]

The PI3K/AKT pathway mediated by phosphatidylinositol-3-kinase (PI3K) and protein kinase B (also known as AKT, threonine-protein kinase) is mainly associated with neuroprotection through prosurvival genes activation, stimulation of the antiapoptotic proteins expression and inhibition of proapoptotic proteins.[7] Mammalian target of rapamycin (mTOR), the most important regulator of ribosome biogenesis and protein translation, is one of the PI3K/AKT cascade components.[8] BDNF-activated TrkB/PI3K/AKT/mTOR signaling cascade is involved in synaptogenesis and synaptic plasticity.[9] NGF is also proved to be involved in synaptogenesis[10] and synaptic plasticity,[3] but there is no data that these NGF functions being mediated by the PI3K/AKT/mTOR cascade.

The pathway mediated by MAPK is involved in neuroprotection, as well as PI3K/AKT.[11] However, the MAPK/ERK cascade may also be involved in neuronal death under pathological conditions.[12] The differences in MAPK/ERK signaling activation dynamics were shown to determine whether the effect of the cascade being neuroprotective or neurotoxic.[13,14]

In addition, the MAPK/ERK signaling cascade is responsible for cell differentiation and proliferation.[6] BDNF/TrkB/MAPK/ERK-mediated activation of the transcription factor cAMP response element-binding protein (CREB) is known to play an important role in the maintenance of synaptic plasticity.[15,16] Although the NGF/TrkA/MAPK/ERK pathway is also associated with CREB activation,[17] there is only a few literature evidence confirming NGF-mediated synaptic plasticity in the hippocampus being associated with CREB.[18]

The pathway mediated by PLCγ1/protein kinase C supports the synaptic plasticity,[6] regulates axon growth and participates in MAPK pathway activation,[19] as well as in the internalization of the ligand/receptor complex into the signal endosome.[20]

In addition, the neurotrophins interact with low-affinity p75 receptors, which depending on conditions serve as Trk coreceptors enhancing the mediated effects or stimulate apoptosis.[21] The p75NTR receptor regulates three major signaling pathways: prosurvival nuclear factor kappa B (NF-κB) cascade, proapoptotic Jun kinase cascade and ceramide cascade which has been shown to promote both apoptotic and prosurvival effects.[6]

NGF and BDNF and their Trk receptors are distributed in the organism in a different way.

Cholinergic neurons are the main target of NGF in the brain.[22] The cholinergic system plays an important role in the processes of learning, memory, and attention, which determines the need for NGF to maintain cognitive functions.[23] The highest level of NGF and TrkA receptors expression is observed in the cortex and hippocampus, that is, in the areas innervated by basal forebrain cholinergic neurons.[24] In addition, NGF is expressed in the hypothalamus and brain stem, regulating the function of noradrenergic nuclei and thus participating in the modulation of the hypothalamic–pituitary–adrenal axis activity.[25]

Unlike NGF, BDNF is expressed in nearly all brain areas, mostly in the hippocampus, cerebral cortex, striatum, hypothalamus, cerebellum, and brain stem[26] providing trophic support to serotonergic, dopaminergic, GABA-ergic, and cholinergic neurons.[27] BDNF is involved in neuroplasticity maintenance not only at the morphological level but at the functional level as well, participating in the modulation of ion channels, including AMPA and NMDA receptors, sodium and potassium channels, transient receptor potential channels.[28] The involvement of BDNF in LTP, which underlies learning and memory, is well-known.[29]

A number of neurological and mental disorders, as well as metabolic diseases, is associated with neurotrophin deficiency.[25,30–34] Thus, in the Alzheimer's pathogenesis impairment of NGF trophic support of cholinergic neurons in the basal forebrain is of great importance.[35] NGF content decrease in the blood plasma and in the substantia nigra is observed in Parkinson's disease.[36,37] BDNF is also involved in the pathogenesis of the Alzheimer's and Parkinson's diseases, and, in addition, in the pathogenesis of amyotrophic lateral sclerosis.[30]

The NGF role in neuroprotection and neuroregeneration in ischemic and traumatic brain injuries is well-known.[31,38,39] An inverse relationship was found between the NGF content in the blood or cerebrospinal fluid on

the first day after the injury and the severity of the neurological outcome.[40–42] Like NGF, BDNF stimulates protective and neuroreparative effects in ischemic injuries and brain injuries.[43–45]

Both NGF and BDNF are involved in the diabetes mellitus pathogenesis, which is associated with their role in support of the viability and function of pancreatic β-cells and the ability to stimulate insulin secretion in response to increased glucose level.[46,47] Interestingly, the pancreatic β-cell dysfunction in diabetes is associated with decreased PI3K/AKT, but not MAPK/ERK signaling activity.[48,49]

A large amount of clinical and experimental data has been accumulated on the BDNF involvement in the pathophysiology of depression.[50,51] BDNF plays a key role in neuroplasticity in the hippocampus and prefrontal cortex, the violation of which underlies the pathophysiology of the disease.[52,53] The antidepressant properties of BDNF was found to be due to the activation of both PI3K/AKT and MAPK/ERK signaling cascades, the two mediate support for neuroplasticity by neurogenesis, synaptogenesis, and synaptic plasticity regulation.[9,18,54] There is little evidence that depression is also associated with a deficiency of NGF.[55]

In connection with the foregoing, NGF and BDNF attract much attention worldwide as a basis for the development of the new drugs. The use of native neurotrophins in the clinic was unsuccessful due to pharmacokinetic restrictions and severe side effects, such as neuropathic pain, dramatic weight loss, and aberrant nervous fiber sprouting.[56] To solve the problem of side effects, pharmaceutical companies and research groups offer selective activation of individual postreceptor signaling pathways by (1) blocking the neurotrophin sites respected to the side effects with monoclonal antibodies, or (2) using the muteins, that is, mutant neurotrophins with mutations in these sites. So, a monoclonal antibody against the C-terminus of NGF was created, that preserved the neuroprotective activity in complex with NGF, but lost the neuritogenic and differentiation activities. Moreover, MAPK signaling was depraved.[57] In 2009, the recombinant mutein NGFK34A, K36A, E35A/F7A, H84A, R103A was described, which retained neuroprotective activity with reduced neuritogenesis.[58] In 2012, the recombinant mutant NGFP61S/R100E was developed, which prevented neurodegeneration in animal models of Alzheimer's disease possessing reduced pain activity and decreased ERK signaling.[59]

The creation of drugs based on neurotrophins requires an integrated approach aimed both at the improvement of the pharmacokinetic characteristics and solvation of the side effects problem. It seems optimal to create a small molecule that would reproduce the therapeutic effects of the native protein without its side effects.

A number of scientific groups have been developing low-molecular-weight mimetics of the neurotrophins.

2 | LOW-MOLECULAR MIMETICS OF NGF

2.1 | Linear peptide analogs

The first low-molecular-weight analogs of NGF were obtained in 1990 in the University of California (San Francisco, CA, USA).[60] As the tertiary NGF structure was unknown at that time, its hydrophilic primary sequence sections (located on the protein surface) with a high degree of cross-species conservation (probably responsible for the main biological functions) were assumed to be crucial for interaction with the receptor, that was used for mimetic design. According to the hypothesis, three NGF regions were selected (amino acid residues 28–38, 66–76, and 99–106), and linear peptides containing 3 to 11 amino acid residues were synthesized based on their structures.

The peptides with amino acid sequences from NGF region 28–38 only selectively inhibited NGF-induced neurite growth in a primary culture of sensory neurons at millimolar concentrations in a dose-dependent manner with the shortest active one, peptide KGKE (C5) consisting of four hydrophilic residues (Figure 2).

These peptides were proved not to affect the binding of NGF to its high-affinity receptors by radioligand studies. In 1991, McDonald et al.[61] published a three-dimensional (3D) structure of the NGF homodimer obtained from X-ray diffraction data. The compound C5 turned out to correspond to the β-turn of the NGF loop 1.

	NGF sequence ^{28}ATDIKGKEVT^{38}V	IC_{50}(mM)
C1:	**ATDIKGKEVTV**	1.1
C2:	**DIKGKEVTV**	1.2
random C2:	**DVEITVKKG**	no inhibition detected (ND)
C10:	**ATDIKG**	(ND)
C6:	**DIKG**	(ND)
C5:	**KGKE**	1.0
(V)C5:	**KVKE**	(ND)
C7:	**KGK**	(ND)
C12:	**GKE**	(ND)
C14:	**VTV**	2.7

FIGURE 2 Linear peptide analogs of NGF on the base of region ^{28}A–^{38}V.[60] Peptide codes in the left column, peptide sequences (bold) in the central column. Here and below, one-letter amino acid code used, unless otherwise stated. NGF, nerve growth factor

2.2 | Monomeric and dimeric cyclopeptide analogs

In 1995, the first cyclic monomeric peptide analogs of the NGF β-turns containing loops were created by the Saragovi H. U. research group (McGill University, Canada)[62] on the assumption such compounds being able to reproduce the 3D structure of the corresponding neurotrophin sections, unlike the linear peptides.

Cyclized monomers (Figure 3) with the KGKE sequence inhibited the neuritogenic activity of NGF at concentrations 100 times lower than the corresponding linear peptide. Among the designed cyclopeptides, the mimetics of the neurotrophin loop 4 were the most active binding to TrkA with an affinity of 10^{-7} M and displacing NGF from these receptors.

In 1997, Longo et al.[63] created both monomeric and dimeric cyclic peptide mimetics of the NGF loops 1, 2, and 4 (Figure 4). The first was inactive, while dimeric compounds had agonistic activity. The mimetics of loop 1 containing the KGKE sequence at a concentration of 10^{-6} M possessed the most pronounced neurotrophic activity in the primary culture of sensory neurons.

Their neurotrophic effect was unaffected by the Trk receptor antagonist K252a, but was blocked by antibodies to p75 receptors, besides no effect was observed in neurons lacking p75 receptors. This suggests these compounds be ligands of p75 receptors, but not of TrkA.

2.3 | Nonpeptide mimetics, p75 receptor modulators

Subsequently, the attention was focused on the NGF loop 1 supposedly involved in the interaction with the p75 receptor. The pharmacophore hypothesis based on the identification of the common structural elements in the loop 1 regions of NGF and NT3 was formulated, stating the side radicals of hydrophobic, positively charged, and neutral amino acid residues as well as subsequent hydrogen bond donor and acceptor with the appropriate space location to be crucial for the receptor interaction. Four residues of the NGF loop 1 were used to construct the pharmacophore. In 2006, based on the hypothesis a focused library of 800 compounds being potential structural analogs of the NGF loop 1 was obtained by in silico screening with Accelrys (USA), Catalyst, and InsightII programs among ca. 800,000 compounds taken from a number of chemical libraries (Chapman & Hall /CRC, National Cancer Institute's anticancer compound library, libraries of companies Maybridge [England], Asinex [Russia], InterBioScreen [Russia], Chemstar [USA], Comgenex [Hungary], Timtec [Germany], Sigma [USA]). Furthermore, 60 compounds were selected by visual analysis using the criteria of steric similarity and maximum functional groups mobility, with 35 of them being commercially available and only 23 being water-soluble.[64] The neurotrophic activity of the latest compounds was studied in vitro in primary cultures of mouse hippocampal neurons and chicken embryos dorsal

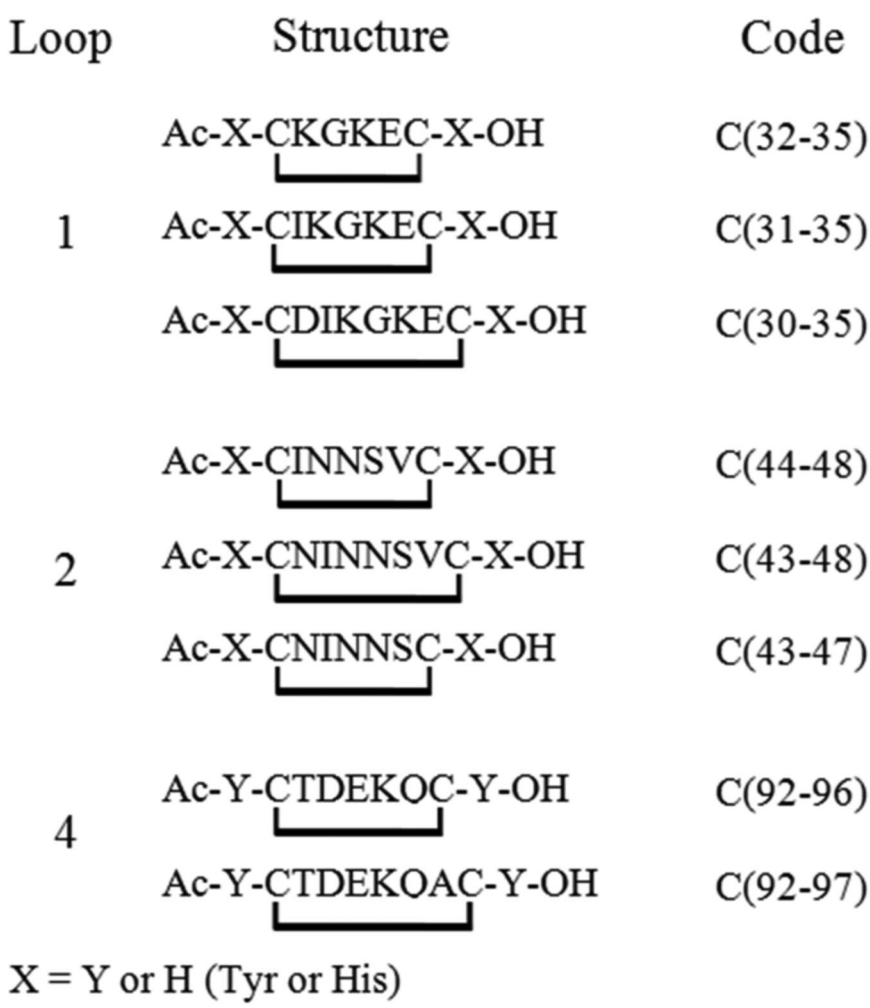


FIGURE 3 Monomeric cyclic peptide analogs of NGF[62] on the base of loops 1, 2, and 4 structures. Peptide code (right column) contains a number of loop amino acid residues. S–S bond cyclization shown by the line. NGF, nerve growth factor

root ganglion (DRG) neurons by means of morphological analysis, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and measurements of GAP43 protein content. As a result, four molecules active in nanomolar concentrations were identified[64] with the maximum effect at 5 nM comparable with the one of NGF and EC_{50} of 0.1–0.3 nM. Two molecules, LM11A-24 and LM11A-31 (Figure 5), were selected for further study following the Lipinsky drug similarity criteria[65] and calculated ability to penetrate through the blood–brain barrier.[66,67] LM11A-24 and LM11A-31 were found to interact selectively with p75 receptors in concentrations of 0.1–10 nM by enzyme-linked immunosorbent assay (ELISA) analysis of competitive binding of the compounds and NGF to recombinant chimeric TrkA-Fc or p75-Fc receptors.

The assumption that LM11A-24 and LM11A-31 effects being through direct interaction with p75NTR was confirmed by the inability of the compounds to elicit prosurvival activity in $p75NTR^{-/-}$ mice hippocampal neurons and by inhibition of their prosurvival effects with antibody targeted to the neurotrophin binding domain of p75NTR. Compounds LM11A are modulators of the receptors that activate signaling associated with neuroprotection and inhibit signaling leading to apoptosis. The neurotrophic effect of compounds LM11A was mediated by NF-κB-signaling cascade of p75 receptors, as shown in an inhibitory assay.

Peptide	Structure	Loop	% NGF max	Conc. With max. activity μM
L29	CTDIKGKEPen-NH_2	1	20	125
L43	PenNINNSC-NH_2	2	18	125
L92	PenTDEKQAC-NH_2	4	15	500
P7	IPenKGKEVCT-NH_2	1	75	250
P8	DPenIKGKEVCT-NH_2		40	500
P9	DPenIKGKEVTCV-NH_2		50	300
P10	TPenDIKGKEVTCV-NH_2		30	2000
P11	TPenTDIKGKEVTCV-NH_2		35	1000
Dimeric P7	IPenKGKEVCT-NH_2 / IPenKGKEVCT-NH_2		70-80	125

FIGURE 4 Monomeric and dimeric cyclic analogs of NGF on the base of loops 1, 2, and 4 sequences.[63] Peptide code (left column) contains numbers of loop amino acid residues. Pen penicillamine residue. S–S bond cyclization shown by the line. NGF, nerve growth factor

As was shown in mature oligodendrocytes expressing p75 and lacking TrkA receptors that undergo apoptosis under the influence of NGF or proNGF, LM11A-24 and LM11A-31 did not cause p75-mediated cell death. In addition, LM11A-24 and LM11A-31 inhibited proNGF-induced apoptosis and binding of proNGF to p75.

LM11A-24 and LM11A-31 was found to prevent the neuronal death and β-amyloid induced damage of neurites and synapses at concentrations of 10^{-7} M in vitro in a cell model of Alzheimer's disease in primary cultures of mouse hippocampal, cortical, and septal neurons.[68]

LM11A-31 was active in a number of models of neurodegenerative diseases at systemic administration. So, in a genetic mouse model of Alzheimer's disease (mice with overexpression of APP) at chronic oral administration (50 mg/kg, 3 months) the compound prevented the working memory deficit development in the Y-maze and the object recognition tests, significantly reduced dystrophy of neurites in basal forebrain cholinergic system, hippocampus, and cortex,[69] as well as inhibited neuroinflammation.[70] It also suppressed age-related basal forebrain cholinergic neuron degeneration at chronic oral administration at a dose of 50 mg/kg in mice.[71] LM11A-31 (50 mg/kg, orally, 4 weeks) reduced retinal blood vessel permeability by inhibition of inflammation and the RhoA kinase pathway in a model of streptozotocin diabetes in mice.[72] In a rat brain injury model, LM11A-31 (33.3 μM intranasally, 14 days) stimulated neurogenesis in the hippocampal dentate gyrus, increasing proliferative activity, progenitor cell survival and the number of mature neurons, and improved impaired spatial memory in the Morris water maze.[73] In addition, LM11A-31 did not cause hyperalgesia when administered intraperitoneally at a dose of 20 mg/kg. The compound (200 mg/kg ip) was found to have no convulsive or anticonvulsant activity in a model of pilocarpine-induced seizures in rats.[74] Currently, LM11A-31 is at Phase 2 of clinical trials as an oral agent for the treatment of Alzheimer's disease (https://clinicaltrials.gov/ct2/show/NCT03069014).

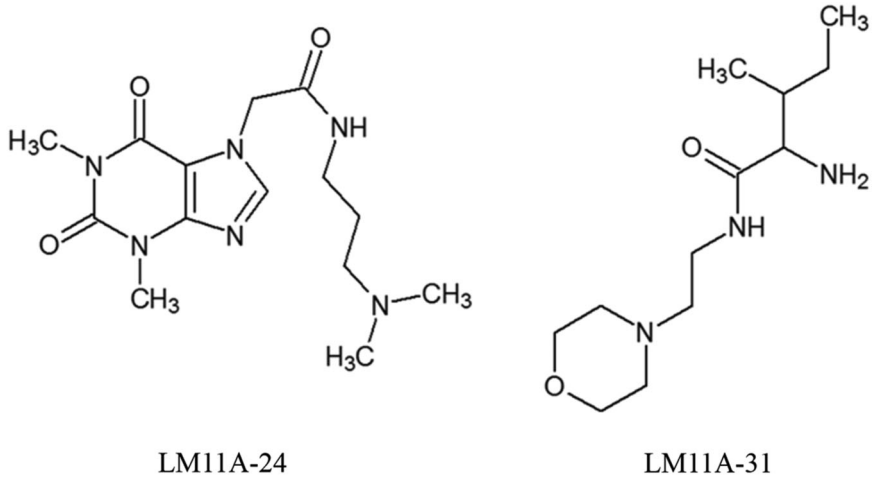


FIGURE 5 Nonpeptide mimetics of NGF-p75 receptor modulators.[64] NGF, nerve growth factor

2.4 | Nonpeptide mimetics, TrkA agonists

In 1999, Saragovi et al designed and synthesized about 60 nonpeptide compounds[75] based on the pharmacophores of the 5C3 monoclonal antibody to the NGF-binding site of the human TrkA receptor[76] and peptide cyclic mimetics of the NGF loop 4 β-turn.[62] Of these, proteolytically stable and water-soluble compound D3 with a mass of 580 Da (Figure 6) was selected in vitro screening for the ability to potentiate the NGF neurotrophic activity at a suboptimal concentration (10 pM) in the NIH-3T3 (p75 − TrkA+) cell line. The compound was proved to be a selective ligand of TrkA receptors without interaction with p75 receptors in the cytofluorometric analysis of the binding of biotin-labeled D3 to rat neuroblastoma B104 cells (p75 + TrkA−) and 4–3.6 (p75 + TrkA+) and in the direct ELISA. Further investigations with the cytofluorometric analysis of the competitive binding of D3 and mAb 5C3 with TrkA on 4–3.6 cells and the competitive ELISA revealed D3 to bind dose-dependently to the same TrkA site as mAb 5C3 with Kdca (2 μM). D3 also inhibited the 5C3 monoclonal antibodies binding to the recombinant TrkA-ECD receptor with Kd calculated on the basis of IC_{50} being also 2 μM. Notably, compound D3 did not block the formation of the NGF complex with TrkA-ECD, however, stabilized the TrkA–TrkA homodimer.

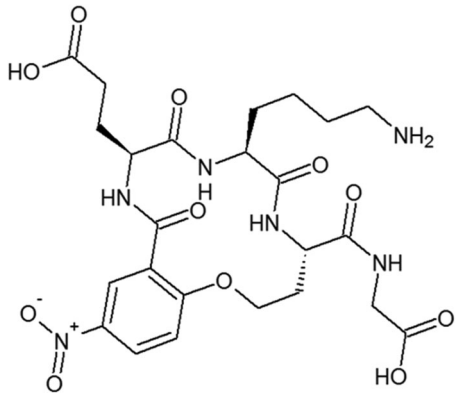


FIGURE 6 Nonpeptide mimetic of NGF compound D3, TrkA agonist.[75] NGF, nerve growth factor

The neurotrophic and differentiating effects of D3 were demonstrated in vitro at a concentration of 10×10^{-6} M in the culture of primary DRG rat neurons.

In old rats, D3 administered intracerebrally (2.8 μg/day/rat for 14 days) had a neuroprotective effect in the cholinergic neurons of the basal forebrain and improved spatial memory in the Morris water maze. Intracerebral administration was used to compare the effects of the compound with those of NGF. D3 possessed better pharmacokinetic properties than full-sized neurotrophin with the severity of the therapeutic effects being comparable with the one of murine 2.5S NGF (1.4 μg/day/rat for 14 days).[77] In a genetic mouse model of Alzheimer's disease (10 and 40 μg/day icv, 14 days) compound D3 significantly improved learning ability and short-term memory in the Morris water maze and reduced the content of β-amyloid in the cerebral cortex, while no effect on learning ability and short-term memory was in the wild-type mice.[78]

A positive effect on the composition and quality of tear fluid was revealed both at acute and chronic administration of D3 (in the form of eye drops) in a scopolamine-induced model of dry eye in rats.[79] There are no literature data on the effects of D3 at systemic administration that may be explained by the instability of D3 in biological fluids due to the presence of a meta-nitrobenzoylamide fragment in the molecule.

To date, compound D3 is at Phase 3 of clinical trials as an ophthalmic solution for the treatment of keratoconjunctivitis (dry eye syndrome; https://clinicaltrials.gov/ct2/show/study/NCT03925727).

2.5 | Oligopeptide analogs, TrkA agonists

In 2008, peptide mimetics of NGF containing the sequences of the loops 1 and 4 (LIL4, 17 residues) and these sequences together with the N-terminal fragment of the neurotrophin (NL1L4, 38 amino acid residues) were created in Rita Levi-Montalcini laboratory[80] (Figure 7).

Three NGF sites interacting with TrkA as shown by mutagenesis and structural studies were selected for the design, namely, fragments of the N-terminus (His^4-Asp^{24}), loop 1 (Thr^{29}-Lys^{34}), and loop 4 (Asp^{92}-Gln^{95}). The latter ones were connected by a tripeptide linker ThrGlyAla to reproduce the appropriate distance in the designed peptides. Loop fragments were cyclized to fix the conformation, for which four cysteine residues were introduced into the structures followed by disulfide bridges formation. Thus, trivalent mimetics of NGF monomer were obtained, which showed agonistic activity. These peptides were demonstrated to induce phosphorylation of TrkA, but not TrkB receptors (immunoblotting assay, PC12 cells, and culture of rat cerebellar granular neurons, respectively) and to possess NGF-like differentiating activity in a culture of embryonic chick DRG neurons at concentrations of $3–6 \times 10^{-6}$ M. In addition, compound L1L4 caused differentiation of the PC12 cells at concentrations of $3–6 \times 10^{-6}$ M, in contrast to NL1L4. The peptide with a higher in vitro activity, L1L4 increased the reduced pain threshold and decreased the intensity of gliosis in the spinal cord in a rat peripheral neuropathy model at central intrathecal administration (37.5 μg/h for 7 days).[80,81] Nevertheless, these peptides still have significant molecular weight and pharmacokinetic problems of a full-sized protein.

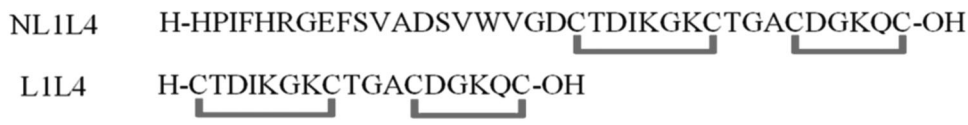


FIGURE 7 Cyclopeptide mimetics of NGF protomers.[80] Peptide code in the left column. NL1L4 consists of N-terminal peptide and fragments of the loops 1 and 4 constrained with S–S bonds. L1L4 contains a loop's cyclic fragments only. S–S bond cyclization shown by the line. NGF, nerve growth factor

2.6 | Nonpeptide mimetics, TrkA agonists

In 2012, a library of peptide chain β-turn mimetics created on the basis of a 3D bicyclic template modified with the side chains of amino acid residues was used to design non-peptide NGF agonists at the Chemistry Department of the Florence University (Italy). About 150 molecules were selected in virtual screening for complementarity with the NGF-binding pocket at the fifth domain of the TrkA receptor.[82] Then, the compound named MT2 was selected from these molecules based on the results of in vitro screening for neurotrophic activity in PC12 cells and on the ability to stimulate proliferation of PC3 prostate carcinoma cells (TrkA+, p75−; Figure 8). Compound MT2 possessed neurotrophic activity at concentrations of $0.1–100 \times 10^{-6}$ M and interacted with TrkA receptors, as was established by radioligand analysis for the displacement of labeled NGF with cold MT2 in PC12 and NIH-3T3 cell lines. MT2 was shown to induce phosphorylation of TrkA receptor tyrosine 490 and to activate the Akt and MAP kinase intracellular cascades in PC12 cells. At concentrations of $10–20 \times 10^{-6}$ M MT2 also had differentiating activity in a primary culture of rat DRG neurons, additionally to neurotrophic one. At concentrations of ca. 10^{-5} M MT2 was found to counteract amyloidogenesis and to increase the viability of neurons in a cellular model of Alzheimer's disease with excessive amyloidogenesis and subsequent cell death caused by the addition of anti-NGF antibodies to a culture of rat hippocampal neurons. Activation of the MAPK pathway influenced by MT2 suggests the latter to have such side effects of NGF as hyperalgesia.

2.7 | Dimeric dipeptide mimetics

A neurotrophin molecule contains loop-like structures protruding in the solvent, which are considered as active sites for receptor binding. In addition, neurotrophins have a symmetric homodimeric structure necessary for interaction with the receptor and its dimerization. The smallest peptide analogs of neurotrophins were obtained at the V. V. Zakusov Institute of Pharmacology. They contain the most exposed dipeptide sequence from the β-turn of the corresponding loop, most often its central fragment, which for geometric reasons can deeply penetrate into the receptor binding zone to provide the best receptor recognition, and the bioisostere of the preceding amino acid residue-free from N-terminal amino group and its positive charge, that provides resistance to hydrolysis by aminopeptidases. The dimeric structure is reproduced by an oligomethylene diamide (commonly hexamethylenediamide) head-to-head spacer. Despite the spacer being much shorter than the distance between the loops of the same type in the neurotrophin structure and the active conformation of peptides being not fixed by cyclization, dimeric dipeptide mimetics were active in a large number of pharmacological tests. The presence of several loops in native neurotrophin suggests that different loops and their mimetics may be responsible for different biological effects.

The dimeric dipeptide mimetics GK-6 (bis(*N*-aminohexanoyl-glycyl-L-lysine) hexamethylenediamide),[83] GTS-115 (bis(*N*-γ-hydroxybutyryl-L-lysyl-L-histidine) hexamethylenediamide),[84] and GK-2 (bis(*N*-monosuccinyl-L-glutamyl-L-lysine) hexamethylenediamide)[85] were designed based on the structures of β-turns of loops 1 (Ile^{31}-Lys^{32}-Gly^{33}-Lys^{34}),

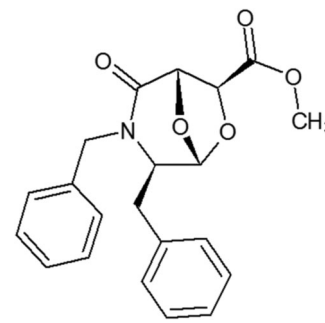


FIGURE 8 Nonpeptide mimetic NGF, compound MT-2–TrkA agonist.[82]
NGF, nerve growth factor

3 (Ser[73]-Lys[74]-His[75]-Trp[76]), and 4 (Asp[93]-Glu[94]-Lys[95]-Gln[96]) of NGF, respectively. The mimetic structure preserved the most exposed β-turn dipeptide fragment, while the previous amino acid residue was replaced by the corresponding bioisostere: succinic acid for Asp, aminohexanoic acid for Lys, and γ-oxybutyric acid residue for Ser (Figure 9).

All dipeptide mimetics of NGF-activated TrkA, herein the mimetics of the NGF loops 1 and 3 (GK-6 and GTS-115, respectively) activated both PI3K/AKT and MAPK/ERK,[84,86] and GK-2, mimetic of the loop 4 selectively activated the PI3K/AKT cascade[83] according to Western blot analysis.

GK-6 and GTS-115 caused differentiation in the PC12 cells,[83,84] while GK-2 was inactive.[83]

All the dipeptides had neuroprotective activity at a concentration of 10^{-8} M in a model of oxidative stress on the HT22 line hippocampal cells, the GK-2 effect severity being comparable in with that of NGF (10^{-9} M), while GK-6 and GTS-115 having less activity (40% relative to NGF).[84,86] The neuroprotective activity of GK-2 was confirmed in glutamate, MPTP and 6-OHDA toxicity experiments both on immortalized and primary rodent neurons, as well as on the SH-SY5Y line human neuroblastoma cells.[85,87]

The dipeptides GTS-115 and GK-2 showed neuroprotective activity in vivo in a model of ischemic stroke in rats caused by transient occlusion of the middle cerebral artery at subchronic ip administration, reducing the volume of ischemic damage by 23 and 45%, respectively, and improving neurological status.[88] The mimetic of loop 1, GK-6, was inactive in the experiment. Notably, GK-2 was effective at the start of administration 1, 4, 6, 8, and even 24 h after surgery in this model.[89] The authors explain the restoration effects of GK-2 firstly administered 24 h after ischemic stroke by neuroregenerative properties of the dipeptide due to the activation of neurogenesis. GK-2 was revealed to stimulate neurogenesis in hippocampus and striatum in experimental ischemic stroke.[90]

Neuroprotective effects of GK-2 were also demonstrated in a number of other experimental models.

GK-2 (1 mg/kg ip) reduced the volume of cortical infarction by 62% at subchronic administration in a model of ischemic stroke in rats caused by bilateral photothrombosis of the cerebral cortex and completely prevented the development of retrograde amnesia in the conditioned passive avoidance test.[91]

In a model of incomplete global ischemia in rats caused by bilateral permanent occlusion of the common carotid arteries, GK-2 (1 mg/kg ip) completely prevented the animals death at subchronic administration (against 40% death in the active control group) and improved the viability of brain cortex cells.[92]

In a model of complete global ischemia in rats caused by cardiac arrest with further resuscitation, GK-2 (0.5 mg/kg ip) when administered 30 min after surgery and then every 24 h for next 3 days accelerated the restoration of the neurological status of animals and prevented damage to Purkinje cells of the cerebellum and hippocampal pyramidal neurons in the early postresuscitative period (7 days after surgery). In the long-term

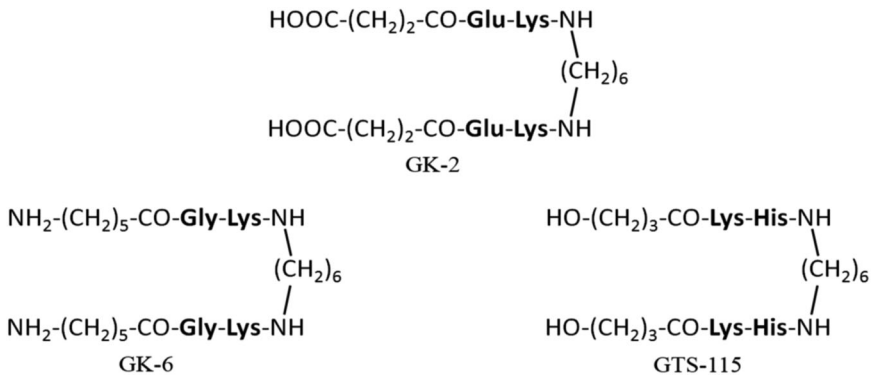


FIGURE 9 Dimeric dipeptide mimetic of NGF loop 1 (GK-6),[83] loop 3 (GTS-115),[84] and loop 4 (GK-2).[85] Dipeptide sequences corresponding to the most externally exposed sites of NGF loop's β-turns are shown in bold. Head-to-head dimerization, no cyclization in the structures. Amino acid residues presented in three-letter code. NGF, nerve growth factor

postresuscitative period (14 days after surgery), GK-2 completely prevented the death of hippocampal neurons and approximately halved the death of cerebellum Purkinje cells.[93]

The dipeptide GK-2 revealed antiparkinsonian effects.[94] In a model of haloperidol catalepsy in rats, GK-2 reduced the catalepsy severity by 80%–90% at ip administration at doses of 0.01–5 mg/kg 24 h before haloperidol and remained active at oral administration at doses of 5 and 10 mg/kg. In a model of MPTP-induced Parkinsonism syndrome in mice GK-2 once administered 24 h before MPTP (1 mg/kg ip), statistically significantly reduced the severity of oligokinesia and rigidity, while at subchronic administration after MPTP it completely prevented the development of a rigidity symptom. GK-2 almost completely prevented the development of apomorphine-induced rotations in rats at subchronic administration (1 mg/kg ip, total 7 injections, start 1 h after surgery) in a model of parkinsonism caused by unilateral 6-OHDA administration in the striatum. GK-2 was active in Alzheimer's disease models in rats.[95] In a septohippocampal transection model GK-2 (1 mg/kg ip every 48 h, the first administration 2 h after surgery, 7 injections) prevented impairment of habituation in rats in an open field test with a therapeutic effect of about 70%. In the cholinergic deficiency model in rats caused by the long-term administration of scopolamine, GK-2 (0.2 mg/kg ip every 24 h for 10 days) almost completely prevented impaired spatial learning ability in the Morris maze. In a model of Alzheimer's disease in rats induced by intracerebroventricular streptozotocin administration, GK-2 (0.5 mg/kg ip every 48 h for 14 days) completely eliminated the spatial memory deficit development in the Morris water maze test. Antidiabetic activity of GK-2 was detected in a model of streptozotocin type 2 diabetes in C57Bl/6 mice.[96] GK-2 (0.5 mg/kg ip 14 days before and 16 days after injection of streptozotocin) had a significant antihyperglycemic effect, that persisted even 44 days after the end of administration.[97] GK-2 also restored the bodyweight of diabetic mice, which was reduced relative to passive control.

The most important side effects of full-sized NGF are hyperalgesia[98] and weight loss.[99] In contrast to NGF, the dipeptide mimetic of the loop 4, GK-2, activating PI3K/AKT selectively, exhibits pronounced analgesic activity in the rat tail-flick test at doses of 1.0 and 2.0 mg/kg (ip), statistically significantly increased the pain threshold 24 h after administration.[83] At the same time, GK-6 and GTS-115 being the mimetics of the loops 1 and 3, respectively, and activating both PI3K/AKT and MAPK/ERK pathways alike NGF, had algesic activity, lowering the pain threshold in rats 1 and 24 h after administration.[83,84]

Dipeptides GK-2, GK-6, and GTS-115 caused no decrease in rat bodyweight at chronic ip administration at the most active doses (0.5, 2.0, and 1.0 mg/kg, respectively).[83,84]

Thus, the use of dimeric dipeptide mimetics of individual NGF loops allowed not only to overcome the pharmacokinetic problems of full-sized neurotrophin but also to separate its useful pharmacological activities from undesirable side effects, as exemplified by GK-2. Preclinical studies of GK-2 as a potential drug for the treatment of poststroke conditions have been completed successfully (https://4science.ru/lot/2015-14-N08-0051). The lyophilized injection dosage form was developed (RU Patent 2678203, 2019). The dipeptide was shown to cross the blood–brain barrier and to be almost nontoxic.[100]

Thus, information on conserved hydrophilic sites in NGF primary structure, the spatial structure of neurotrophin loops, and pharmacophores of the monoclonal antibody to NGF were used for the design of the NGF mimetics. Mimetics were constructed on the template of the peptide chain β-turn taking into account the structure of spatial pharmacophores of NGF, virtual screening of chemical libraries with the selection of compounds spatially similar to β-turns of loops and screening for in vitro activity were hold. Another strategy was to use the most exposed tripeptide sequence of loop-like structures usually being the fragments in the β-turn for the design.

3 | LOW-MOLECULAR MIMETICS OF BDNF

3.1 | Monomeric and dimeric cyclopeptide analogs of individual loops

The first low-molecular-weight BDNF mimetics were obtained by Richard Hughes' research group from the University of Melbourne (Australia).[101] The 3D structure of BDNF was unknown at the research started, so the Hughes

group constructed it using homologous modeling based on the 3D structure of NGF, described by McDonald et al.[61] The 3D structure of BDNF such obtained was later confirmed by X-ray diffraction studies of Robinson et al.[102] By that time, chimeric NGF with the second loop of BDNF implanted was known to acquire the ability to bind to TrkB receptors.[103] In this regard, the attention of the researchers was drawn to loop 2, which they defined as Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51} sequence. Cyclic peptides conformationally limited by disulfide bridges of cysteine residues were constructed using Hyperchem simulation. Four compounds containing from 12 to 6 BDNF amino acid residues that were theoretically conformationally close to loop 2 were selected using the Polack-Ribiere algorithm and the MM + force field (L2–12, L2–10, L2–8, L2–6; Figure 10).

Designed peptides were obtained by solid-state synthesis followed by oxidation of cysteine residues with dimethyl sulfoxide at pH 8.0.

The peptides synthesized were revealed to inhibit the survival mediated by BDNF in a concentration-dependent manner in a study of the sensory neurons survival in chicken embryos. The maximum effect of all the peptides was observed at micromolar concentration. The severity of the effect for peptides L2–12 and L2–10 was 40%, for peptide L2–8 50%, for peptide L2–6 27%. The corresponding linear peptides were inactive. All cyclopeptides did not inhibit the action of NGF. So for the first time, specific competitive TrkB antagonists were obtained.

In the next work,[104] the authors constructed dimeric di- and tricyclic peptide mimetics of the loop 2 with agonistic activity based on the most active L2–8 cyclopeptide with the help of the Sybyl program. The mimetics included bicyclic dimeric peptides connected with disulfide or amide bond and tricyclic dimeric peptides connected both by amide and disulfide bonds (Figure 11). The introduction of cysteine residues performed disulfide dimerization into the L2–8 sequence. The amide bond dimerization was carried out by the introduction of lysine and glutamic acid as the C-terminal residues with a further combination of the side functional groups by an amide bond.

The site of dimerizing bond introduction was determined based on the distances between the corresponding amino acid residues in loop 2.

The designed compounds were synthesized by the solid-phase method in combination with the techniques of the amide bond (γ-Glu–ε-Lys) formation in partially protected products and the disulfide bond formation by oxidation of cysteine residues.

The effects of bicyclic dimers were studied in primary cultures of chick embryo sensory neurons. Compounds 2, 4, 5, and 6 showed a concentration-dependent increase in neuronal survival. Among the bicycles, compound 5 was the most active with $EC_{50} = 10^{-10}$ M and effect severity of 30% from that of BDNF. Tricyclic compound 6 was the most active of all the compounds (35% of BDNF effect; $EC_{50} = 10^{-11}$ M). All substances concentration-dependently inhibited the effects of BDNF on survival, thus being its partial antagonist–agonists. Compound 3 possessed only antagonistic properties.

To reproduce the BDNF spatial structure containing three pairs of loops involved in receptor binding, the Hughes group obtained dimeric mimetics of BDNF loops along with the monomeric analogs.[105] Both intrachain and interchain dimers were constructed consisting of the fragments from the same polypeptide chain and different polypeptide chains, respectively (Figure 12). In the first ones (heterodimers) loops 1 and 2, 2 and 4 were combined.

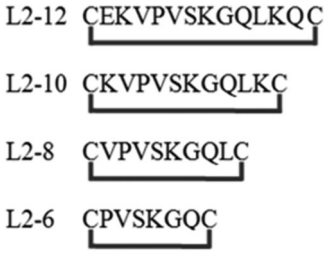


FIGURE 10 Cyclic mimetics of the BDNF loop 2 with antagonistic activity.[101] Peptides codes (located left) contain a loop index and a number of amino acid residues. S–S bond cyclization shown by the line. BDNF, brain-derived neurotrophic factor

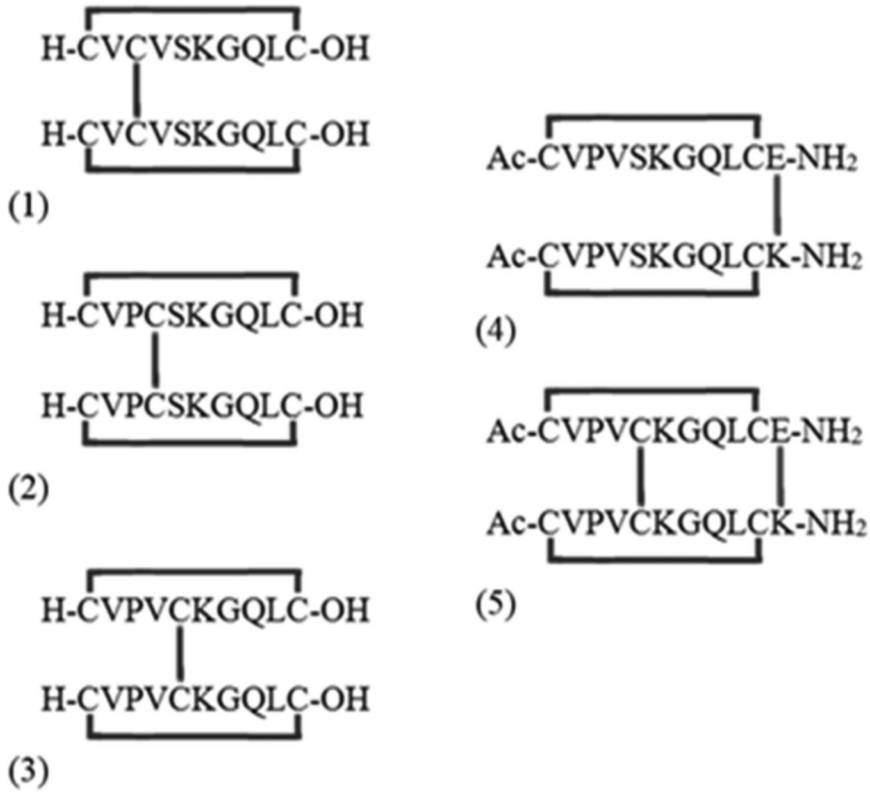


FIGURE 11 The structures of bi- and tricyclic mimetics of BDNF loops with agonistic and antagonistic activity[104] with the free (1–3) and blocked (4 and 5) terminals. Cyclization and dimerization bonds shown by lines. BDNF, brain-derived neurotrophic factor

In the second ones (homodimers) combination of 2 and 2′ loops, 4 and 4′ loops of different BDNF polypeptide chains was used. The cysteine S–S bridges position was determined both visually and using the Sybyl 6.4 program for minimization of the disturbances introduced into the structure.

Monomeric peptides inhibited the effects of BDNF on the chick embryos sensory neurons survival in a concentration-dependent manner. L1 was the least active (10^{-5} M, 30% inhibition). Other monomeric peptides were active even at a concentration of 10^{-7} M with L2a possessing the highest inhibitory activity. The compound was active in the range of 10^{-9}–10^{-5} M with a maximum effect of 45% at a concentration of 10^{-5} M. L4a and L4b, mimetics of the loop 4, significantly inhibited the effects of BDNF at a concentration of 10^{-7} M with the greatest effect 49% and 41%, respectively, at 10^{-5} M.

Almost all dimeric mimetics had inhibitory activity as well. Only the homodimeric bicyclic peptide L4b–L4b possessed agonistic activity, being able to increase the survival of sensory neurons at a concentration of 10^{-5} M.

To summarize, the Australian scientists demonstrated both monomeric monocyclic peptides and heterodimeric bicyclic peptides based on loops 1, 2, and 4 to be BDNF inhibitors, while homodimeric bicyclic peptides being BDNF agonists.

Thus, low-molecular-weight BDNF mimetics with agonistic activity were obtained both based on the neurotrophin loops 2[104] and 4.[105]

Being low-molecular-weight analogs of BDNF, these compounds still were too big and complex in structure for further development as medicines. In this regard, the Hughes group created a cyclic proteolytically stable BDNF mimetic, which was a cyclic pentapeptide, cyclo(-D-Pro-Ala-Lys-Lys-Arg-), being not TrkB but p75 neurotrophin

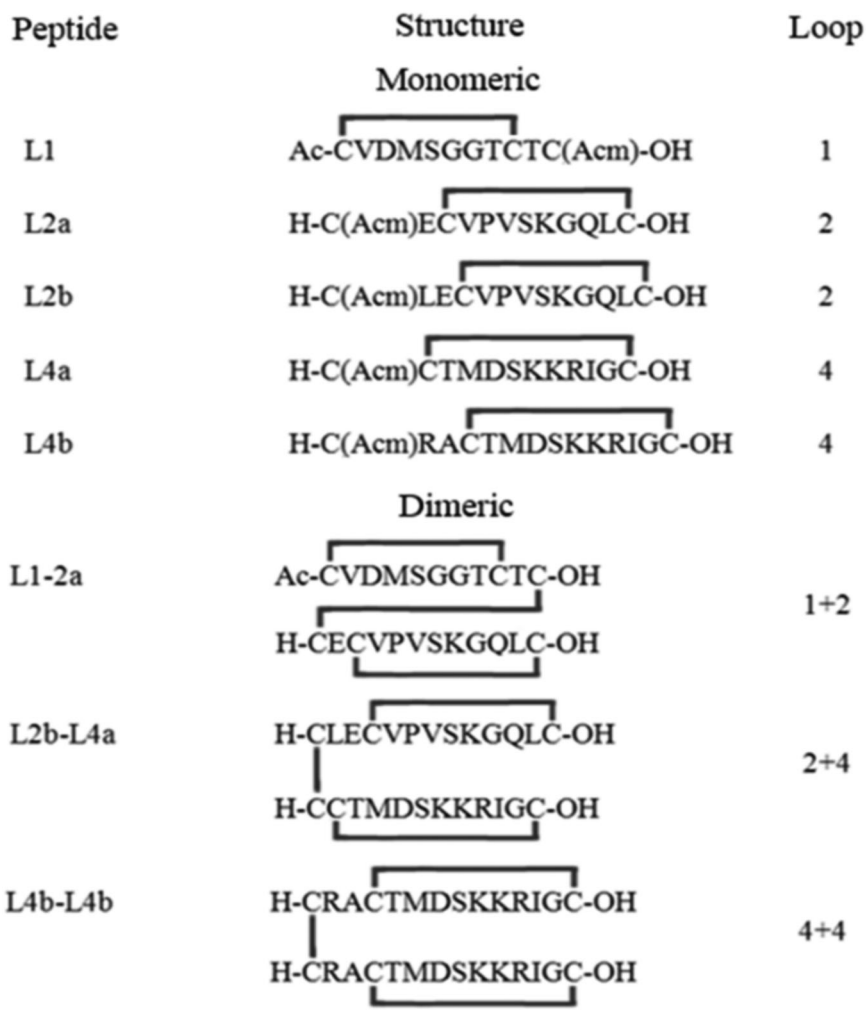


FIGURE 12 Monomeric and dimeric peptide mimetics of the BDNF loops 1, 2, and 4.[105] Peptide codes contain loop number. S–S bond cyclization shown by the line. Acm, acetamidomethyl protecting group; BDNF, brain-derived neurotrophic factor

receptor ligand, however.[106] The cyclopeptide was constructed based on the site-directed mutagenesis data on the key role of the tripeptide sequence -Lys-Lys-Arg- of BDNF loop 4 in interaction with the p75 receptor.[107]

The cyclopeptide construction was achieved using Sybyl 6.4 and Hyperchem 4.0 programs. The tripeptide sequence Lys^{94}-Lys^{95}-Arg^{96} of the BDNF loop 4 was got from the 3D BDNF structure obtained by homologous modeling.[104] Next, a wide range of conformational constraints was examined for the selection of amino acid residues to be added between the N- and C-ends of the tripeptide sequence. As such, pairs of Gly, Ala, and Pro (including *D*-forms), as well as β-amino acids and other ω-alkyl amino acids of various lengths, were considered. Each structure was optimized to a local minimum of conformational energy in the AMBER force field in Hyperchem. Promising cyclopeptides were subjected to further conformational studies in Hyperchem using the Conformation search application, the torsion angles of the main chain were changed randomly, and the resulting structures were minimized in energy again. Unique low-energy conformations with the standard deviation of the α- and β-carbon atoms from the corresponding atoms of the natural tripeptide less than 0.4 Å were used for further work.

The visual modeling resulted in 57 cyclopeptides. Minimization of energy reduced their number to 9, only two peptides left after conformational analysis and comparison with the natural tripeptide, namely, 6-aminohexanoyl containing tetrapeptide (cyclo-(Ahx-Lys-Lys-Arg)) and D-Pro- containing pentapeptide (cyclo-(D-Pro-Ala-Lys-Lys-Arg)). Both were synthesized by solid-phase peptide synthesis, followed by cyclization in solution. The cyclopentapeptide (cyclo-DPAKKR) was the most active in the sensory neurons survival experiments on the culture of 8-day-old chicken embryos with 38% of BDNF activity at 10^{-6} M and 68% at 10^{-4} M, respectively. It increased the effect of BDNF being inactive on an NGF-dependent culture of neurons.

The cyclopentapeptide did not affect TrkB and its downstream signaling pathways (MAPK), as shown by Western blot analysis. Its interaction with the p75 receptor was confirmed by the promotion of peripheral myelination of nerve fibers in vitro and in vivo.[108] The p75 receptor is known to be involved in this process, while TrkB activation inhibits myelination. In vitro experiments were conducted on a culture of NGF-dependent neurons of the DRG of newborn Sprague Dawley rats. Myelination was determined by Western blot analysis with monoclonal antibodies to myelin-associated glycoprotein (MAG) and to myelin basic protein (MBP) and histochemically. The peptide was active at concentrations of 10^{-8}–10^{-7} M. In addition, neither cyclopentapeptide nor BDNF was active on DRG neurons from p75NTR$^{-/-}$ mice. This demonstrates the p75 expression to be absolutely necessary for the promyelinating action of both BDNF and cyclo-DPAKKR. In vivo experiments were performed on Sprague Dawley rats by subcutaneous (sc) injection of 3.2 μg of cyclo-DPAKKR followed by sciatic nerve extraction and analyses of the main regulator of myelination NRG1-type III expression, as well as by MAG and MBP Western blot analysis. The cyclopentapeptide was shown to increase NRG1-type III expression and myelination, while BDNF was inactive. Cyclo-DPAKKR or compounds alike enhancing myelination through the p75 receptor only can be used to treat peripheral demyelinating diseases.

Unlike the pentapeptide, the tricyclic dimeric peptide 6 (see Figure 9), selectively activating the TrkB receptor, selectively enhanced the central myelination of neurons (0.1–100 nM in vitro),[109] which can be useful in the treatment of such diseases, like multiple sclerosis.

To improve the pharmacokinetic properties, cyclo-(D-Pro-Ala-Lys-Lys-Arg) was hydrophobized by replacement of Ala with Lys and the introduction of an n-alkylacyl group into the ω-amino group of this lysine.[110] The effective concentration of the pentadecanoyl derivative of cyclo-DPKKKR decreased by two orders in vitro (pEC_{20BDNF} = 9.1), while its stability in rat plasma and the ability to penetrate through model biological membranes increased.

3.2 | Nonpeptide analogs of the loop 2

The Longo group[111] developed nonpeptide analogs of the BDNF loop 2, based onimportance of the SKGOL site for the manifestation of neurotrophin activity, data obtained using chimeric NGF/BDNF proteins.[103,112] In accordance with the spatial structure of the site, Longo advanced a pharmacophore hypothesis used for virtual screening. The postulated pharmacophore had 35 conformers, for each of them, more than a million compounds from the libraries Asinex, AMRI, Interbioscreen, Sigma-Aldrich, Timtec, Chemstar were screened in silico. Virtual screening resulted in 1855 candidates fitted with a pharmacophore conformer with a free energy of less than 10 kcal/mol. This amount was reduced to 14 by application of such criteria as the absence of a volume interfering the interaction with the receptor, molecule flexibility, molecular weight between 500 and 650 Da, and Lipinsky criteria. Compounds poorly fitted these criteria and hardly overlapped the pharmacophore were excluded. Seven among the compounds left were commercially available; an in vitro assay for them was performed on hippocampal mouse E16 neurons. Four of them having neurotrophic activity received codes LM22A-1, LM22A-2, LM22A-3, and LM22A-4 (Figure 13). They showed 80%–89% of BDNF activity with an EC_{50} of 200–500 pM.

All the compounds proved to be selective TrkB receptor partial agonists, as shown by inhibitory assay with K252a and Western blot analysis of TrkB^{Y490}p on cells expressing TrkB or TrkA or TrkC only. Selected due to the structural simplicity and the modernization possibility, compound 4, 1,3,5-benzene tricarboxylic acid tri

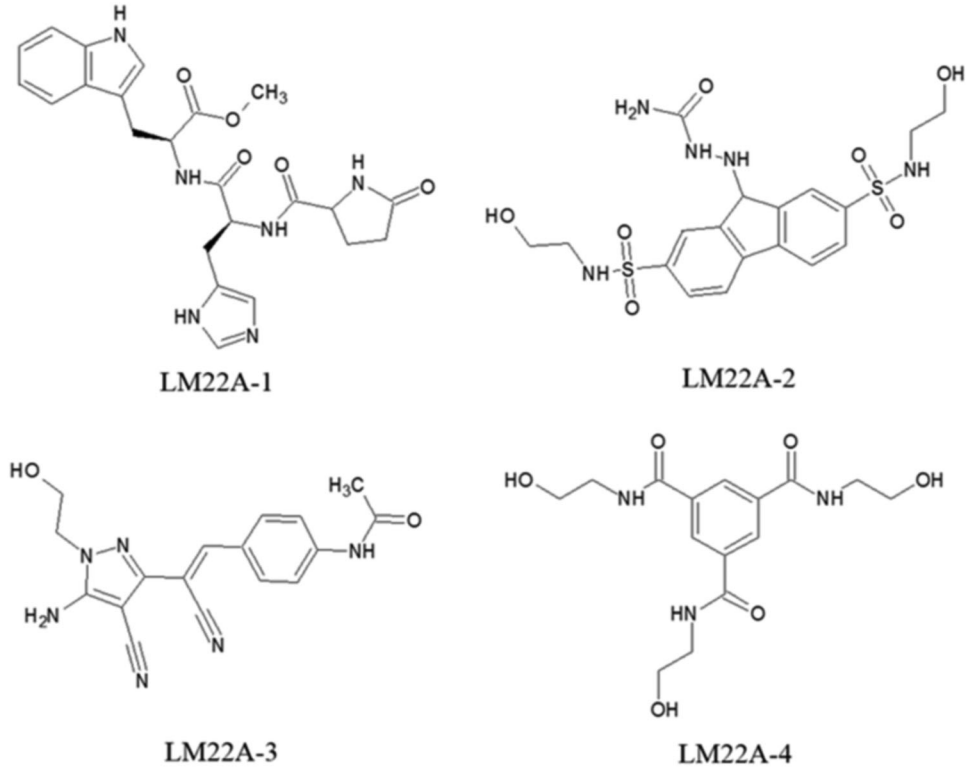


FIGURE 13 Nonpeptide mimetics of BDNF loop 2 with agonistic activity.[111] Compound's codes located under the structures. BDNF, brain-derived neurotrophic factor

(hydroxyethylamide), was active in cell models of Alzheimer's, Huntington's, and Parkinson's diseases at a concentration of 0.5 μM. Compound 4 contributed to the motor functions restoration after experimental brain injury in rats after a 2-week administration at a dose of 3.4 μg.

The effectiveness of compound LM22A-4 was demonstrated in models of Rett syndrome in vitro (0.5 μM) and in vivo (150 mg/kg ip, mice).[111,113]

Moreover, the Japanese researchers from Hiroshima University showed[114] the LM22A-4 to regulate the cement blocks differentiation similar to BDNF, the processes being carried out through the TrkB-ERK/AKT signal cascade. This may provide a new medicinal method for periodontal tissue regeneration and the treatment of periodontal disease.

3.3 | Linear tetrapeptide analogs of BDNF

A research group from the New York State Institute of Fundamental Research on Developmental Disorders received five therapeutically promising tetrapeptides B1–B5 corresponding to the sequences 6–9, 71–74, 94–97, 72–75, 115–118 of human BDNF[115] (Figure 14).

The peptide sequences were identified as epitopes of monoclonal antibodies to active BDNF sites. To block charges of terminal amino acid, the peptides were acylated at the N-terminus and amidated at the C-terminus, m as follows: B1 (Ac-RRGF-$CONH_2$), B2 (Ac-IDKR-$CONH_2$), B3 (Ac-SKKR-CONH2), B4 (Ac-DKRH-$CONH_2$), and B5 (IKRG-CONH2).

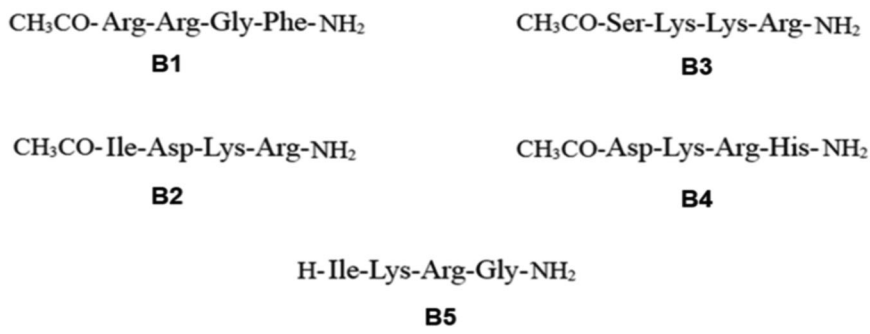


FIGURE 14 BDNF linear tetrapeptide mimetics with agonistic activity.[115] Peptide's codes located under the structures. Three-letter amino acid code used. BDNF, brain-derived neurotrophic factor

All the peptides caused moderate TrkB receptor activation (Y706 phosphorylation), which was blocked by K252a, an inhibitor of the Trk family. The peptides B3 and B5 were the most active functioning as partial BDNF agonists/antagonists. They increased the survival of murine primary E18 hippocampal neurons (0.1–1 μM) and caused the expression of MAP2 neuronal markers, β-III tubulin, NTM, and NeuN at a concentration of 0.8 nM, similar to BDNF. Unlike B5, B3 potentiated the BDNF effect on protection neurons from oxidative stress. The authors suggested compound B3 to have additional signaling different from that of BDNF, activating other receptors than TrkB. The authors concluded these peptides to be more promising drugs than the Hughes' dimeric cyclopeptides because of the lower molecular weight and better biological barriers penetration, and more promising than the Longo's nonpeptide mimetics due to the metabolization to natural amino acids.

3.3.1 | 7,8-Dihydroxyflavone

7,8-Dihydroxyflavon (7,8-DHF; Figure 15) is a low-molecular-weight TrkB agonist (254 Da) that currently being developed as a potential antidepressant in Emory University (Atlanta, USA).[116,117]

7,8-DHF was selected from 2000 biologically active compounds from the Spectrum Collection Library. First, 66 compounds targeting either TrkB receptors or their postreceptor pathways were selected based on in vitro screening for neuroprotective activity with TrkB expressing cells.[118] Five of them were derivatives of flavone or its modifications. 7,8-DHF was the most active in the concentration range of 10–250 nM. Protective effects of the compound in the oxygen–glucose deprivation tests in the mouse cell line even exceeded the ones of BDNF. 7,8-DHF also demonstrated neuroprotective activity in the primary culture of human neurons under oxidative stress.[118] Unlike other flavone derivatives, 7,8-DHF was proved to cause TrkB phosphorylation in hippocampal neurons by immunofluorescence analysis and Western blot. 7,8-DHF also activated AKT and ERK1/2 that was confirmed by inhibitory analysis. 7,8-DHF-induced phosphorylation of TrkB was blocked by a K252a, Trk receptor inhibitor. The neuroprotective activity of 7,8-DHF was significantly blocked by MEK or PI3K inhibitors in vitro.

7,8-DHF was active in a number of in vivo models of neurological and mental disorders, such as Parkinson's (per os, 14 days, neuroprotection),[119] Huntington's (5 mg/kg, 12 weeks, improvement of motor and cognitive functions),[120] and Alzheimer's (5 mg/kg per os, 4 months, stimulation of synaptogenesis, improvement of cognitive functions)[121] diseases, multiple sclerosis (5 mg/kg ip, 28 days, reduction of demyelination and axonal loss in CNS),[122] Down's syndrome (5 mg/kg sc, 7 weeks, stimulation of hippocampal neurogenesis, improvement of cognitive functions),[123] schizophrenia (5 mg/kg ip, 14 days, improvement of hippocampal synaptic plasticity and cognitive functions),[124] ischemic strokes (5 mg/kg ip, acute, improvement of the neurological score, reduction of

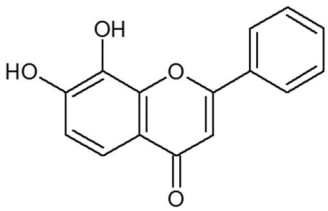


FIGURE 15 Structure of 7,8-dihydroxyflavone[118]

cerebral infarct volume by about 40%),[109] and traumatic brain injury (5 mg/kg ip, 2 weeks, stimulation of neurogenesis).[125]

The antidepressant properties of 7,8-DHF were established. 7,8-DHF (10–20 mg/kg ip, 28 days) attenuated agedonia in the sucrose preference test, restored reduced TrkB phosphorylation, and activated the expression of synaptic markers PSD95 and synaptophysin in a model of chronic moderate stress in mice.[116] 7,8-DHF (5 mg/kg per os, 21 days) significantly increased the proliferative activity of neuronal stem cells in the dentate gyrus of the hippocampus in adult mice.[117]

Thus, the data on site-directed mutagenesis and chimerization of NGF/BDNF, a search among epitopes of monoclonal antibodies to BDNF active sites, randomized screening among chemical libraries based on pharmacophore proposed as a result of site-directed mutagenesis data analysis, and also in vitro broad-scale screening were used for the design of BDNF mimetics.

3.4 | Dimeric dipeptide mimetics

The fundamentally different approach based on the use of central fragments of loop β-turns as the most exposed outward and, therefore, most accessible for interaction with the receptor was used for mimetic design at the V. V. Zakusov Research Institute of Pharmacology. The strategy resulted in the smallest of all possible peptide analogs of BDNF dipeptides.

The dipeptide mimetics GSB-214 (bis(*N*-monosuccinyl-L-methionyl-L-serine) heptamethylenediamide), GTS-201 (bis(*N*-hexanoyl-L-seryl-L-lysine) hexamethylenediamide), and GSB-106 (bis(*N*-monosuccinyl-L-seryl-L-lysine) hexamethylenediamide) were designed based on the β-turn structures of loop 1 (-Asp^{30}-**Met^{31}-Ser^{32}**-Gly^{33}-), loop 2 (-Val^{44}-**Ser^{45}-Lys^{46}**-Gly^{47}-), and loop 4 (-Asp^{93}-**Ser^{94}-Lys^{95}**-Lys^{96}-) of BDNF, respectively.[126–128] The central β-turn dipeptide fragment was retained in the structure of mimetics, and the previous amino acid residue was replaced by its bioisostere: Asp was replaced by the residue of succinic acid, Val—by the residue of hexanoic acid (Figure 16).

By Western blot analysis with antibodies to phosphorylated and nonphosphorylated kinases all the compounds were demonstrated to activate the BDNF-specific tyrosine kinase receptor TrkB, but with different patterns of postreceptor signaling, namely activation of PI3K /AKT and MAPK/ERK pathways.[128,129]

Like a full-sized neurotrophin, the loop 4 mimetic, GSB-106, activated both PI3K/AKT and MAPK/ERK,[130] while the loop 1 mimetic, GSB-214, and the loop 2 mimetic, GSB-201, selectively activated PI3K/AKT[129] and MAPK / ERK,[128] respectively.

All BDNF dipeptide mimetics exhibited neuroprotective activity at concentrations of 10^{-6}–10^{-8} M protecting HT22 hippocampal neurons from H_2O_2-induced oxidative stress, with a maximum effect of ~50% of the one of BDNF (10^{-9} M).[126,128,131] The effect of the most active compound, GSB-106, was confirmed on HT22 cells under glutamate toxicity conditions and on SH-SY5Y line human neuroblastoma cells under conditions of 6-OHDA-induced toxicity[131]; in both cases, at a concentration of 10^{-7} M the dipeptide showed the same activity level as BDNF.

The dipeptide mimetics of the BDNF loops 4 and 1 (GSB-106 and GSB-214), the first activating both PI3K/AKT and MAPK/ERK pathways, while the second activating PI3K/AKT only, showed neuroprotective activity in vivo in

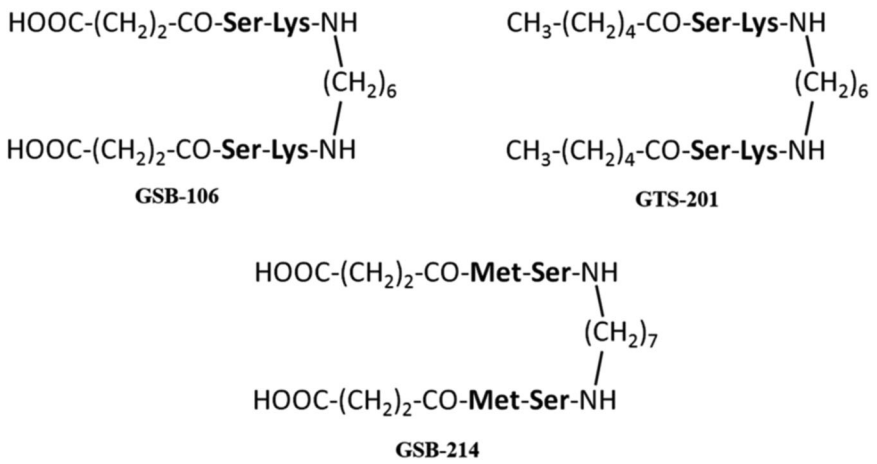


FIGURE 16 Dimeric dipeptide mimetics of BDNF loop 1 (GSB-214),[126] loop 2 (GTS-201),[128] and loop 4 (GSB-106).[126] Dipeptide sequences corresponding to the central fragments of the BDNF loop's β-turns depicted in bold. Three-letter amino acid code used. BDNF, brain-derived neurotrophic factor

the model ischemic stroke caused by transient middle cerebral artery occlusion in rats; the compound reduced the cerebral infarct volume by 66% and 28%, respectively.[129] The BDNF loop 2 mimetic, GTS-201, selectively activating MAPK/ERK exhibited no neuroprotective activity under these conditions.

GSB-106 showed antidepressant activity at doses of 0.1 and 1.0 mg/kg ip in the forced swimming test on BALB/c mice, while selective GSB-214 and GTS-201 were inactive.[126,128] The antidepressant activity of nonselective mimetic only suggests that PI3K/AKT and MAPK/ERK should be promoted simultaneously for effect.

The antidepressant properties of GSB-106 were confirmed in outbred mice and rats at acute and subchronic administration ip in the Nomura water wheel test and in the tail suspension test.[132] The antidepressant activity persisted at oral administration in rats at doses of 0.5–5.0 mg/kg for substance[133] and at doses of 0.01–5.0 mg/kg for a tablet dosage form (RF Patent RU2697254 C2, 2019).

The antidepressant activity of GSB-106 in the dosage form was also investigated in a model of a depressive state in C57Bl/6 mice caused by 10-day social stress.[127] The dipeptide administered once (0.1 mg/kg orally, after stress) showed antiahedonic activity by restoration of the impaired preference of sucrose solution in stressed mice.

The ability of GSB-106 (10 mg/kg ip, 5 days) to prevent stress-induced disturbances in hippocampal neurogenesis was completely established in a model of subchronic stress in C57Bl/6 mice caused by contact with a predator.[134] Chronic administration of GSB-106 (1.0 mg/kg ip, 21 days) to BALB/c mice under physiological conditions led to a significant (50%) increase in the synaptophysin (synaptic vesicle membrane protein) content in the hippocampus.[135] The pronounced antidepressant effects of GSB-106 may be explained by its stimulating effect on hippocampal neurogenesis and synaptogenesis.

Dipeptide mimetics of BDNF activating PI3K/AKT exhibited antidiabetic activity at chronic administration,[96] with GSB-214 activating PI3K/AKT only being the most active. Its antihyperglycemic effect persisted for at least 44 days. The effect of the GSB-106, which activates both PI3K/AKT and MAPK/ERK pathways, was less pronounced and persisted for 4 days only. Compound GTS-201 activating only MAPK/ERK was totally inactive.

The PI3K/AKT pathway activation was confirmed to be necessary and sufficiently for antidiabetic activity in experiments with pharmacological inhibitory analysis. The antidiabetic effects of GSB-214 were completely eliminated by LY294002, a specific PI3K/AKT inhibitor.[136] The results for dipeptide mimetics of BDNF are consistent with the data on the PI3K/AKT involvement in the diabetes pathogenesis obtained in the experiments in transgenic mice deficient in PI3K/AKT[137] and with overexpression of PI3K/AKT.[138]

The mimetic of BDNF loop 4, GSB-106, showed analgesic activity in hot plate tests and tail-flick test in rats at intraperitoneal administration at doses of 0.1 and 1.0 mg/kg within 0.5–48 h after injection.[139] Notably, the analgesic activity of morphine preserved no more than 3 h in the same tests.[140] The loop 2 mimetic GTS-201 activating MAPK/ERK only possessed weak analgesic activity, while the mimetic activating PI3K/AKT only was totally inactive.

Thus, the dipeptide BDNF mimetic GSB-106 exhibits CNS effects at systemic administration, including oral administration, likewise the NGF dipeptide dimeric mimetics. Preclinical studies of GSB-106 as a potential antidepressant have been successfully completed (https://4science.ru/project/14-N08-12-0086). A tablet dosage form has been developed (RU Patent 2 697 254 C1, 2018). The dipeptide was demonstrated to cross the blood–brain barrier[141] and to be almost nontoxic.[142]

4 | CONCLUSIONS

Over the past three decades, the development of low-molecular-weight mimetics of neurotrophins both peptide and nonpeptide nature is being carried out on the basis of several strategies. Initially, while the 3D structure of NGF was unknown, the active sites of neurotrophin were searched among cross-species-conserved hydrophilic residues, that is, structurally important fragments located on the protein surface. The discovery of the 3D structure of neurotrophins allowed either to use virtual screening of chemical libraries to obtain nonpeptide analogs based on the similarity to the identified 3D pharmacophores found out to be the β-turns of neurotrophin loops (mimetics LM11A-24, LM11A-31), or to construct nonpeptide mimetics on a cyclic template of β-turn (mimetics MT-2, D3). In addition to analogs of individual loops, mimetics of extended sections of neurotrophins were created, constructed from the pharmacophores of the N-terminal fragment, loops 1 and 4 all located on one side of the homodimer (compound NL1L4). Notably, cyclization of protein loops fragments led to an increase in activity.

The design of the first low-molecular-weight BDNF mimetics was fulfilled based on the variant of the BDNF structure constructed by homology with NGF.

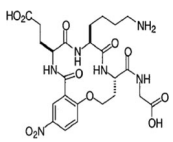
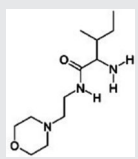
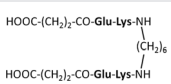
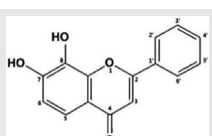
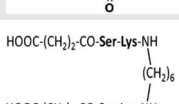
First, monomeric peptide mimetics of the loop 2 were obtained by cysteine residues introduction at the ends of peptide fragment for cyclization by disulfide bonds; next, their dimerized analogs were designed and synthesized. Another strategy resulted from a computer simulation was to obtain a cyclopentapeptide with the introduction of a D-amino acid for better cyclization. Linear tetrapeptide analogs were obtained on the basis of epitopes of monoclonal antibodies to BDNF. Nonpeptide analogs of loop 2 were selected by virtual screening trough chemical libraries of compounds similar to the spatial structure of the SKGOL site, being important for the neurotrophin activity manifestation, as was shown in chimeric NGF/BDNF proteins. Subsequent global-scaled in vitro screening resulted in a very promising mimetic, 7,8-dihydroxyflavone.

Linear dimer dipeptide mimetics of β-turns of NGF and BDNF loops being the shortest fragments and, therefore, the most synthetically available showed high activity without cyclization. The possible explanation is fast conformational transitions in the compounds and receptor selection of complementary β-turn conformations of *N*-acyl dipeptide, that are additionally fixed by an intramolecular hydrogen bond.

The dimeric dipeptide mimetics of NGF and BDNF individual loops activating the corresponding Trk receptors showed different patterns of activation of postreceptor pathways PI3K/AKT and MAPK/ERK that allowed to reveal the contribution of each to different types of pharmacological activity. So, activation of PI3K/AKT or MAPK/ERK is sufficient for the manifestation of neuroprotective activity in vitro. At the same time, activation of PI3K/AKT is necessary for the same activity in vivo, for example, in experimental brain ischemia. Activation of PI3K/AKT is necessary and sufficient for the manifestation of antidiabetic activity. For antidepressant activity, simultaneous activation of PI3K/AKT and MAPK/ERK TrkB receptor signaling pathways is required.

The efforts of various scientific groups to create low-molecular-weight mimetics of neurotrophins led to the production of a number of therapeutically promising compounds (see Table 1). One of them, the p75 receptor

TABLE 1 The therapeutically promising mimetics of NGF and BDNF

Compound			Pharmacological activity in vivo			
Code	Structure	Mechanism	Model	Active doses	Intended application	Development stage
D3		TrkA agonist	Alzheimer's disease, natural aging, keratoconjunctivitis	2 μg (rats), 10 and 40 μg (mice), icv[77,78] 5 μl 1% solution, eye drops) (rats)[79]	Keratoconjunctivitis	Phase 3 of clinical trials (eye drops) https://clinicaltrials.gov/ct2/show/study/NCT03925727
LM11A-31		p75 modulator	Alzheimer's disease, natural aging, brain injury	50 mg/kg, per os (mice),[69,70] 33 μM, intranasal (rats)[73]	Alzheimer's disease	Phase 2 of clinical trials (oral capsules) https://clinicaltrials.gov/ct2/show/NCT03069014
GK-2		TrkA agonist with preferable activation of PI3K/AKT	Neurodegenerative diseases, cerebral ischemia	1 mg/kg ip (rats),[90] 10 mg/kg, per os rats)[94]	Poststroke condition	Preclinical studies (lyophilized injection dosage form) https://4science.ru/lot/2015-14-N08-0051
7,8-Dihydroxyflavone		TrkB agonist	Neurodegenerative diseases, cerebral ischemia, depression	5 mg/kg ip (mice)[122] 5 mg/kg, per os (mice)[117]	Depression	Advanced pharmacological studies
GSB-106		TrkB agonist, activating PI3K/AKT and MAPK/ERK	Cerebral ischemia, depression	0.1 mg/kg ip,[126] 0.1 mg/kg, per os[127]	Depression	Preclinical studies (tablet dosage form) https://4science.ru/project/14-N08-12-0086

Abbreviations: AKT, protein kinase B; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

modulator LM11A-31 showed neuroprotective, neuroregenerative, and cognitotropic activity in models of natural aging, Alzheimer's disease and brain injury in rodents. The compound caused no hyperalgesia and had no convulsive activity. Currently, LM11A-31 is at Phase 2 of clinical trials as an oral dosage form for Alzheimer's disease treatment. Another NGF mimetic, a TrkA D3 receptor agonist, demonstrated neuroprotective and cognitive activity in rodent models of Alzheimer's disease and natural aging at intracerebral administration; it also had therapeutic effects in keratoconjunctivitis models. D3 is undergoing Phase 3 clinical trials as a treatment for keratoconjunctivitis in the form of eye drops.

The dimeric dipeptide mimetic of the NGF loop 4, GK-2 was active in models of Parkinson's disease, Alzheimer's, and stroke at systemic administration, including oral one, and showed neurogenic and synaptogenic activity. At the same time, the dipeptide did not cause an increase in pain sensitivity and weight loss, the main side effects of a full-sized protein. The compound crosses the blood–brain barrier and is almost nontoxic. Dipeptide GK-2 has successfully passed preclinical studies as a potential drug for the treatment of poststroke conditions.

The TrkB receptor agonist 7,8-dihydroxyflavone had a pronounced antidepressant activity and showed a neuroprotective effect in animal models of Parkinson's, Alzheimer's, and Huntington's diseases at oral administration. 7,8-Dihydroxyflavone is undergoing extensive pharmacological studies as a potential antidepressant.

The dimer dipeptide mimetic of the BDNF loop 4, GSB-106, which showed neuroprotective and antidepressant activity in a number of models, is active at systemic administration, including the oral one, does not cause hyperalgesia and weight loss, well penetrates through the blood-brain barrier, being almost nontoxic. Dipeptide GSB-106 has successfully passed preclinical studies as a potential antidepressant.

Thus, to date, two neurotrophin mimetics are undergoing clinical trials, two have successfully completed preclinical studies, and one is at the stage of advanced pharmacological studies.

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