



Novel Technologies for Dipeptide Drugs Design and their Implantation



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Abstract: The article is an overview of author's data obtained in the framework of the project "The Creation of dipeptide preparations" at the V.V. Zakuov Institute of Pharmacology, Moscow, Russia. Advantages of dipeptides over longer peptides consist in that they are orally active owing to higher stability and ability to penetrate biological barriers due to the presence of specific ATP-dependent transporters in enterocytes and blood-brain barrier. Two original approaches for dipeptide drugs design have been developed. Both of them are based on the idea of a leading role of central dipeptide fragment of the peptide chain beta-turn in the peptide-receptor interaction. The first approach, named "peptide drug-based design" represents the transformation of known nonpeptide drug into its dipeptide topological analog. The latter usually corresponds to a beta-turn of some regulatory peptide. The second approach represents the design of tripeptide mimetic of the beta-turn of regulatory peptide or protein. The results of the studies, which led to the discovery of endogenous prototypes of the known non-peptide drugs piracetam and sulpiride, are presented herein. The paper discusses the process, based on the above-mentioned principles, that was used in designing of nontoxic, orally available, highly effective dipeptide drugs: nootropic noopept, dipeptide analog of piracetam; antipsychotic dilept, neurotensin tripeptide analog; selective anxiolytic GB-115, tripeptide analog of CCK-4, and potential neuroprotector GK-2, homodimeric dipeptide analog of NGF.

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1. INTRODUCTION

One of the powerful sources for the design of novel effective medicines are regulatory peptides and proteins [1].

Active site of regulatory peptides and proteins interacting directly with the receptor molecule consists of the limited number of amino acid residues amount of which is determined by the competition between precision of recognition and rate of peptide-receptor complex dissociation [2]. Usually, an active site of regulatory peptides represents a peptide chain β turn. Four residues are involved in β turn, two ($i + 2$, $i + 3$) of which are in the middle of it (Fig. 1). According to geometrical reasons, their side chains immerse most fully into the cavity of the receptor upon peptide-receptor interaction and therefore play a major role in the recognition of whole peptide by receptor. This is a theoretical basis for the dipeptide drug design.

Peptide drugs have obvious advantages over the non-peptide drugs such as high activity, low toxicity since they consist of endogenous amino acids, the absence of severe side effects due to the regulatory action, and lower probability of tolerance or dependence. The superiority of dipeptides over poly- and oligopeptides in many cases is their oral bioavailability due to the ability to penetrate biological barriers and absence of targets bounds for exopeptidases. An important factor is that dipeptides are more technically available in comparison to poly- and oligopeptides.

Traditional strategy of peptidic drugs development is based on the endogenous peptide structure modification. The commonly used approaches are: the searching of the minimum active peptide sequence, amino acid residues modification including the change of

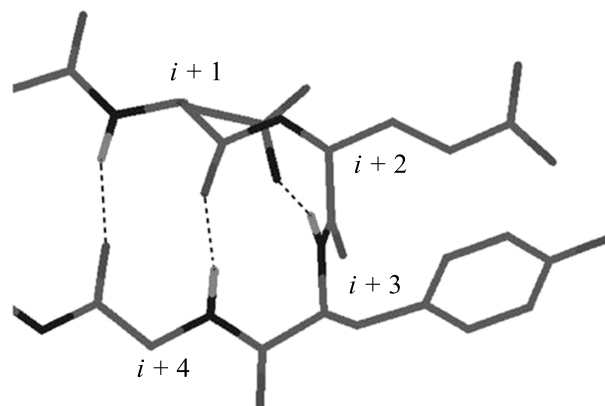


Fig. (1). Conformation of the peptide chain β -turn.

configuration, replacement of peptide bonds with the pseudopeptide bonds, addition of C or N-terminal substitution, synthesis of peptidomimetics.

The new dipeptide drugs construction strategy developed in the V. V. Zakuov Research Institute of Pharmacology represents the evolution from the known non-peptide neuropsychotropic drug to topological similar biologically active peptide [3-5]. This strategy is based on the idea that many known non-peptide neuropsychotropic drugs previously discovered by the screening or heuristics, in fact are the ligands of peptide receptors. The molecular weight of non-peptide neuro psychotropic drugs (usually <500 Da) corresponds to di- or tripeptides that support our assumption. The milestones of the drug-based dipeptide design are the next:

1. selection of the origin non-peptide drug on the base of U-shape of dose-effect dependence that is a pharmacological signs of peptidergic mechanism of action;

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- identification of the "peptide-like" elements in the structure of this drug
- design of the simplest peptide analog based on the identified peptide-like elements;
- testing the target activity of the peptide analog;
- optimization of the analog' structure using the structure-activity relationship (SAR) method;
- searching and identification of the endogenous prototype of the most active peptide analog;
- modification of the analog, taking into consideration the endogenous neuropeptide structure
- selection of the dipeptide for development as a potential medicine.

Using this technology, two dipeptide drugs (nootropic Noopept and neuroleptic Dilept) were developed at the V. V. Zakusov Research Institute of Pharmacology and several drugs are in now progress.

2. NOOPEPT, A DIPEPTIDE NOOTROPIC DRUG

The design of dipeptide cognitive enhancer Noopept was started from the structure of the classical nootropic non-peptide drug, Piracetam [6, 7]. We suggested a peptidergic mechanism of Piracetam based on its pharmacological properties. The next elements of amino acids in its structure were found: Piracetam structure contains a pyrrolidine ring, which can mimic the *side* chain of the pyroglutamic acid or proline, and *N*-acetamide moiety, which can mimic the glycine residue (Fig. 2).

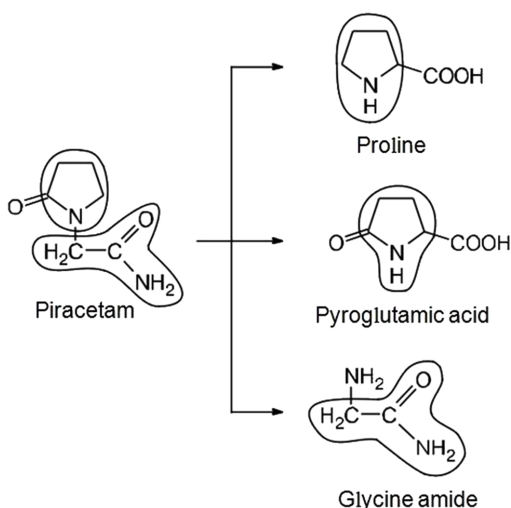


Fig. (2). Structural basics for the design of dipeptide analogs of piracetam.

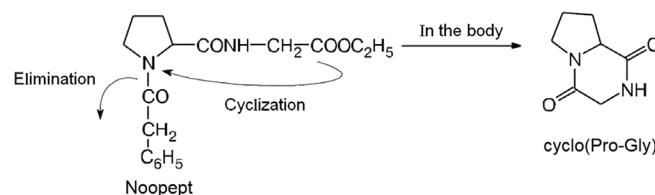
Coming from this assumption, the *p*Glu—Gly—NH₂ dipeptide was designed as the simplest peptide analog of piracetam. This dipeptide was shown to be active in dosage 1,000 times lower than its prototype [6].

The SAR study allowed us to construct the *p*Glu—Asn—NH₂ dipeptide which was 20,000 times more active in comparison with Piracetam [8, 9].

This dipeptide coincides with the *N*-terminal segment of AVP(4-9), the main vasopressin metabolite, the endogenous modulator of recognition and memory processes. In experiments, *in vitro* it was shown that the *p*Glu—Asn—NH₂ dipeptide, like AVP(4-9), demonstrates a neuroprotective activity related to the neurotrophin (NGF) synthesis activation [10]. The results suggest that Piracetam may act via the AVP(4-9) receptors. These results support our hypothesis of the peptidergic mechanism of Piracetam action.

We also used proline as pyrrolidine containing aminoacid for development of another peptide analogs of Piracetam [11, 12]. *N*-acyl moiety was added to proline residue to mimic carbonyl moiety that is present in pyroglutamic acid and is absent in proline Fig. (2).

We designed and synthesized a number of active linear *N*-acylproline-containing dipeptides with the common formulae R—CO—Pro—AAR—X (R—CO is acyl, AAR is amino acid residue, X is a functional group such as NH₂, OMe, OEt) [13-15] and a cyclic dipeptide, cycloprolylglycine (*cyclo* (Pro—Gly)). The last one was coincided topologically with Piracetam most exactly; therefore *cyclo*(Pro—Gly) represents another possible endogenous prototype of Piracetam [16]. After a SAR analysis and consequent design, *N*-phenylacetyl-L-propylglycine ethyl ester was selected as the most active and technologically available compound for the subsequent development as a medicine. This compound was named Noopept (originating from the words "nootropic" and "peptide") [17].



Scheme 1. The *in vivo* formation of *cyclo* (Pro—Gly) as the major metabolite was displayed for Noopept [18].

Later on, we identified *cyclo*(Pro—Gly) in the rat brain as an endogenous neuropeptide [19, 20]. Later it was demonstrated that *cyclo* (Pro—Gly) is a product of the insulin-like growth factor I (IGF I) processing [21]. The *in vivo* investigations of *cyclo* (Pro—Gly) showed that this dipeptide has pharmacological profile resemble that of Piracetam. Like Piracetam, *cyclo* (Pro—Gly) possess nootropic [22], neuroprotective, antihypoxic [23] and anxiolytic [24] activities. Evaluation of the effect on memory phases in passive avoidance test in rats demonstrated that *cyclo* (Pro—Gly) similar to Piracetam is active only when administered before learning. In contrast, Noopept facilitates all memory phases, including the input of information, consolidation, and retrieval. We concluded that Noopept should be considered not only as a prodrug of *cyclo*(Pro—Gly) but also has its own activity. Radioligand investigations identified two types of specific binding sites of [³H] Noopept: high affinity sites with $K_d = 6.85 \cdot 10^{-7}$ M and $B_{max} = 1.6$ pmol/mg of the protein and low affinity sites with $K_d = 7.69 \cdot 10^{-4}$ M and $B_{max} = 73.2$ pmol/mg of the protein. It should be specified that this dissociation constant for the high affinity Noopept binding sites is in agreement with the effective doses of this nootropic agent (0.1—1.0 mg/kg, i.p., or $3 \cdot 10^{-7}$ — $3 \cdot 10^{-6}$ mol/kg).

Pharmacological study of Noopept identified three key types of activities: nootropic, neuroprotective and anxiolytic [25, 26]. Nootropic properties manifested themselves in the ability to reduce memory deficit caused by a wide range of amnesic factors: electroshock (as a model of retrograde amnesia), pretreatment with central muscarinic antagonist scopolamine or prolyne cethyl ester, an original amnesic agent designed in the Institute of Pharmacology (as models of anterograde amnesia). The range of Noopept's effective doses was between 0.1-1.0 mg/kg (depending on the test). For comparison the threshold dose of piracetam is 200-1000 mg/kg. The ratio of effective doses to the toxic ones is known to characterize the safety range of every pharmacological compound. For Noopept with its ED₅₀ 5100 mg/kg and optimal doses 0.5-1.0 mg/kg this ratio is about 10000, while for piracetam (ED₅₀ 8000 mg/kg and optimal doses 300 mg/kg) the safety ratio does not exceed 30. The experiments with active avoidance acquisition have shown an acceleration of the learning skills after administration of

Noopept. Besides effectiveness in these tests of associative learning, Noopept demonstrated the activity in non-associative test of negative learning (the habituation of explorative behavior). Noopept lacked the stimulating or sedative effect. The drug did not display an anti-convulsive or analgesic effects. Even in doses three orders higher than nootropic ones Noopept provoked neither motor discoordination, nor muscle relaxation. Taken together these data are testifying for the specific type of Noopept' nootropic action.

The advantages of Noopept over Piracetam are characterized not only by the quantitative (the lower level of effective doses), but also by qualitative features. While Noopept was shown to facilitate the initial memory trace formation, its storage and retrieval, Piracetam improved the initial phases of memory formation only with no effect on memory retrieval. These differences allow to consider Noopept to be beneficial for the treatment of a greater range of memory-related disorders compared to Piracetam.

Noopept showed effectiveness in several animal models of Alzheimer's disease: olfactory bulbectomy [27], administration of amyloid into Meiner nucleus [28] and intracerebroventricular administration of diabetogenic toxin streptozotocin [29]. Noopept was shown to ameliorate the behavioral consequences of photochemically induced stroke [30] and mechanical trauma of prefrontal cortex [31]. Noopept exhibited pronounced neuroprotective effects. The ability of Noopept to attenuate the severity of oxidative stress was established in neuronal cultures of various types: granular cerebellar cells [32], cortical neuron culture of aborted fetuses with diagnosed Down syndrome [33], PC12 culture [34], SH-SY5Y culture [35]. The ability to enhance superoxide dismutase and catalase activity was shown in the experiment on rats [36] and human blood samples [37].

Noopept's neuroprotective effect was demonstrated on the cellular model of Alzheimer's disease: it attenuated the disturbance of oxidative processes and calcium homeostasis, enhanced the neurogenesis, prevented the tau protein aggregation caused by a fragment of β -amyloid (25-35) [34], eliminated NGF and BDNF deficit caused by intracerebroventricularly administered diabetogenic toxin streptozotocin [29]. Noopept was capable to reduce the cytotoxic effect of aggregated α -synuclein in a cell model of Parkinson's disease [35].

Noopept was shown to dose-dependently increase (0.5-5.0 mg/kg) the time the mice spent in open arms of elevated plus-maze, as well as to increase the number of punished water licks in Vogel test. These effects demonstrate the anxiolytic activity of Noopept [25].

Noopept in the form of tablets (10 mg) has successfully passed clinical trials for treating patients with mild cognitive impairment of cerebro-vascular of posttraumatic origin. It has been present on the market since 2006.

3. DILEPT, A DIPEPTIDE NEUROLEPTIC

The same approach (creation of dipeptide topological analogue of nonpeptidic drug) was used to design a dipeptide neuroleptic. The structure of a non-peptide atypical neuroleptic Sulpiride was selected as starting point on the base of the functional and structure similarity between Sulpiride and neuropeptides such as the bell-shape dose dependence and the presence of amino acid and peptide elements in molecule [38].

The structure of Sulpiride contains a pyrrolidine moiety, analog of the proline side chain, and a substituted phenyl group, mimicking the tyrosine side chain. Furthermore, the Sulpiride molecule has an amide group, which can be considered as a peptide bond analog (Fig. 3). Thus, prolyltyrosine amide was designed as the simplest Sulpiride dipeptide analog. Sulpiride and Pro-Tyr-NH₂ were well superimposed on each other when modeling by Dreiding models or PC-Model 7.0 package were used (Fig. 4). It was shown that the geometry of dipeptide in the pharmacophoric conformation is simi-

lar to that of the central dipeptide segment of the peptide chain β -turn.

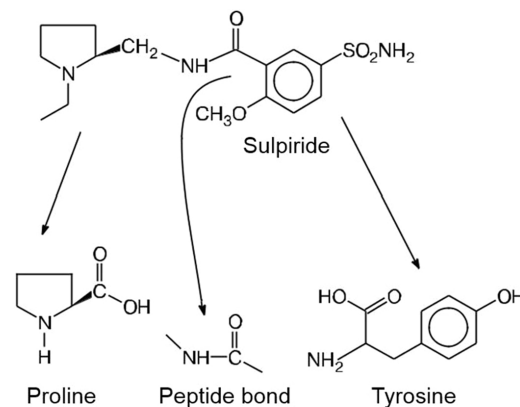


Fig. (3). Peptide structural elements of sulpiride.

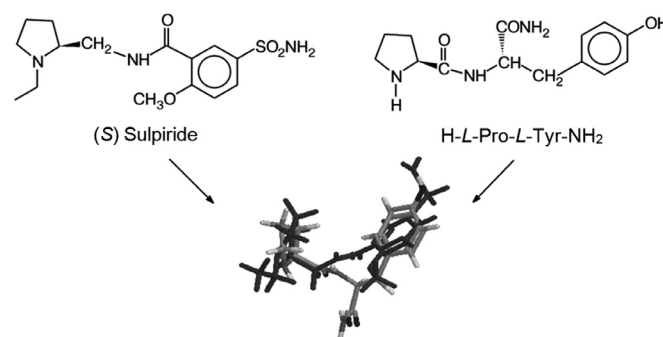


Fig. (4). Superimposition of Sulpiride (dark) and L-Pro-L-Tyr-NH₂ dipeptide molecules in unstrained conformations (PC Model 7.0).

The L-Pro-L-Tyr-NH₂ dipeptide demonstrated the neuroleptic-like activity i.p. in doses 10 times lower than Sulpiride [39].

It should be mentioned that the Pro¹⁰-Tyr¹¹ sequence is presented in the neurotensin - the neuropeptide well-known as an endogenous neuroleptic.

The further design of a dipeptide neuroleptic was based on the NT(8-13) β -turn structure (Fig. 5) postulated as the biological active conformation. In this conformation, the Leu¹³ side chain is proximate with Pro¹⁰. Therefore, we mimicked the Leu¹³ by an *N*-acyl moiety introduced into dipeptide Pro-Tyr-NH₂. Molecular modeling showed that a certain length of this group sufficient to imitate the function of the Leu¹³ side chain.

In fact, the introduction of a six σ -bonds led to 10-times increasing of the activity [40].

By this way, the methyl ester of *N*-caproyl-L-propyl-L-tyrosine was received. This dipeptide was called Dilept from the words "dipeptide" and "neuroleptic". It was selected for extended pharmacological investigations [5].

Dilept was shown to inhibit the apomorphine-induced climbing in mice, ameliorate the DOPA-induced disturbance of water-escape in rats, and decrease the degree of amphetamine-induced hyperlocomotion in mice. It potentiates the effect of barbiturates and exerts a hypothermic effect. These effects of Dilept are manifested in the dose range between 0.4-4 mg kg⁻¹ upon the intraperitoneal administration (i.p.) or 12-20 mg kg⁻¹ upon oral administration (p.o.). At the same time the drug did not induce catalepsy nor did it provoke the myorelaxation or sedation up to doses of 500 mg/kg, which is about 1000 times higher than ED₅₀ in apomorphine-induced climbing test - 0.54 mg/kg [40, 41]. Moreover, Dilept even attenuated the cataleptic effect of haloperidol. In contrary to other neuroleptics,

Dilept improves the cognitive performance in passive avoidance and novel objects recognition tests.

Although screening tests allowed to identify Dilept as a potential new antipsychotic drug, translational models might be more promising to evaluate the possible clinical effect of the new drug. One of the models that have gained a tremendous interest in this respect is the Prepulse Inhibition (PPI) - an operational measure of sensorimotor gating in which a startle reflex is inhibited by a weak stimulus presented prior to the startling stimulus. The deficit in sensorimotor gating is leading to the cognitive fragmentation and thought disorder that is typical for schizophrenia. The disruption of PPI caused by dopamine agonists and N-methyl-D-aspartate antagonists is known to be the most adequate experimental model of this disease. Typical and atypical antipsychotics can restore disrupted PPI and their effectiveness in this test is highly correlated with their clinical potency. Dilept was shown to eliminate the amorphine or ketamine induced prepulse inhibition deficiency [42].

According to E. Richelson (Mayo Foundation, Jacksonville, Florida, USA, unpublished data), Dilept demonstrated specific binding to recombinant rat neurotensin receptor (NT1) with a micromolar dissociation constant.

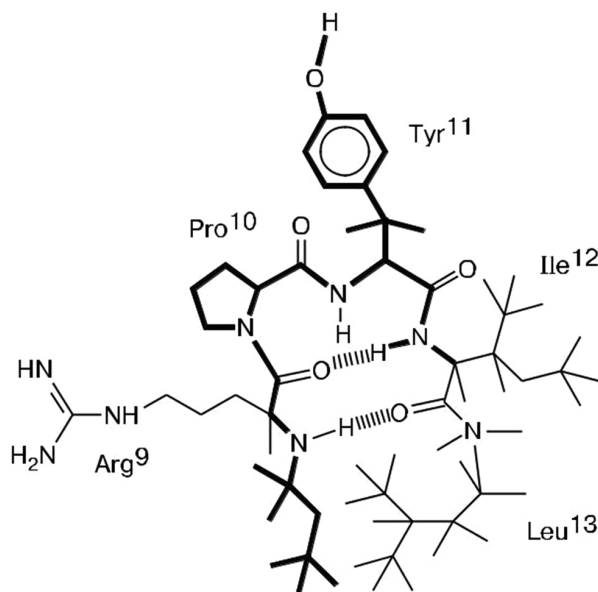


Fig. (5). Superimposition of NT(8-13) in the type I β -turn conformation (thin lines) and *N*-caproylpropyltyrosine amide (bold lines) in Dreiding models.

Comparison of Dilept with typical and atypical neuroleptics demonstrates that Dilept is almost ten times more active than Sulpiride or Clozapine according to the effective doses range, which is close to Haloperidol's one, but, unlike the latter, Dilept does not cause catalepsy even in a dose 1000 times higher than the therapeutic one. Dilept is virtually non-toxic: $LD_{50} > 5000 \text{ mg kg}^{-1}$ (i.p.). The study of Dilept pharmacokinetics in rats demonstrated that the drug is detected in blood plasma for 30 min upon peroral administration; it penetrates the blood brain barrier and can be detected in the brain [43]. Dilept was found to increase the turnover rate of dopamine (DA) selectively in nucleus accumbens, leaving unchanged this parameter in the striatum [44]. The clinical efficacy of both typical and atypical antipsychotics is directly correlated with their effect on D2 receptors in the mesolimbic DA system, while the extrapyramidal symptoms liability of typical antipsychotics is related to blockade of DA transmission in the nigro-striatal system. Therefore, this profile of Dilept allows to propose the antipsychotic effect with presumed lack of extrapyramidal side effect

and tardive dyskinesia. In this respect, Dilept differs from typical antipsychotics dramatically.

Dilept as tablets (20 mg) has passed Phase II of clinical trials. The results confirmed experimental findings: Dilept demonstrates the combination of antipsychotic, pro-cognitive effects and the lack of extrapyramidal side effects.

4. GB 115, A SELECTIVE DIPEPTIDE ANXIOLYTIC

The classical strategy "from the endogenous peptide to the drug" was used for design of selective dipeptide anxiolytic. As the initial design object we have selected the anxiogenic neuropeptide CCK-4.

The design included the following milestones:

- using of the topochemical principle;
- preservation of the side chains of hydrophobic amino acid residues;
- replacement of non-pharmacophoric amino acids by a spacer;
- replacement of pharmacophoric amino acids by their bioisosteres.

We used the variant of topochemical principle [45], according to which a peptide consisting of L-amino acids arranged in a sequence opposite to the native one may have the same activity as the peptide consisting of D-amino acids because the mutual spatial arrangement of the amino acids' moieties is retained in this case.

The application of this topochemical principle to the anxiogenic CCK-4 (Trp-Met-Asp-Phe-NH₂) yielded the retro analog (Phe-Asp-Met-Trp-NH₂) with assumed anxiolytic activity (Fig. 6). Applying concept of the major role of hydrophobic interactions in the peptide—receptor recognition we kept the aromatic amino acid residues, Phe and Trp, while the internal amino acid residues were replaced by the Gly-Gly, resulting in the Phe-Gly-Gly-Trp-NH₂ tetrapeptide (Fig. 6).

Then we replaced the Phe-Gly dipeptide fragment with the topologically equivalent phenylhexanoyl moiety in order to increase the biological stability of the CCK 4 analog. In the process of the whole design, the same distance between the phenyl and indolyl pharmacophoric groups was kept.

Thus, based on the structure of the endogenous anxiogenic tetrapeptide we received the substituted dipeptide, C₆H₅-(CH₂)₅-CO-Gly-Trp-NH₂ (GB-115), with a presumed anxiolytic activity [46] (Fig. 6).

The anxiolytic activity of GB-115 was confirmed in the elevated plus maze, which is the key experimental test for analyzing the effect of drugs on anxiety.

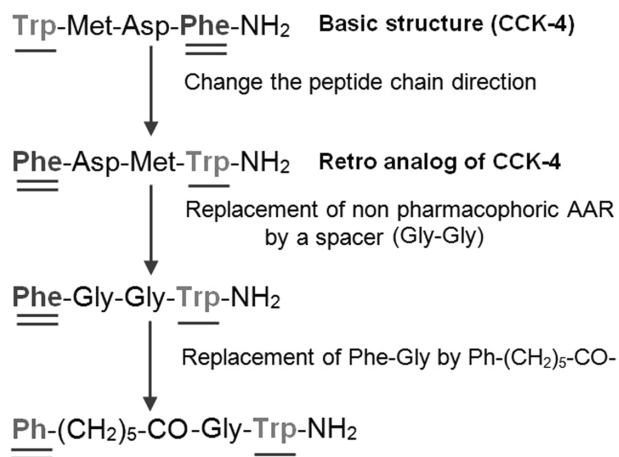


Fig. (6). Design of the GB-115 dipeptide with the anxiolytic activity.

This compound proved to be so effective that it was selected for a further pharmacological investigation [47]. It should be noted that in full agreement with the topochemical principle the D- Trp-based enantiomer exhibited the anxiogenic activity CCK-4. The pharmacophoric similarity of the GB-115 D-enantiomer with CCK-4 was demonstrated using the SYBYL 7.1 software. The structural file for the pharmacophoric conformation of CCK-4 was kindly provided by the laboratory of D. Fourmy (France).

The structure-function analysis has demonstrated that in the series of GB-115 homologs, the activity is present only if the native distance between the hydrophobic (phenyl and indolyl) pharmacophores is retained. A decrease in the distance by even one CH₂ group results in a tenfold decrease in the activity.

GB-115 exhibits anxiolytic activity on animal models in the dose range of 0.002–0.2 mg kg⁻¹ (i.p.) [48]. The effect persists in the case of oral administration (0.1–1 mg kg⁻¹). This dipeptide acts at the anxiety and fear regulation level. This was demonstrated by the pharmacogenetic approach of S. B. Seredenin [49] using stress resistant (C57/Bl6) and stress predisposed (Balb/c) mice in the open field test. The anxiolytic effect of GB-115 is abolished by the CCK-4 tetrapeptide in stress predisposed animals. Meanwhile, the anxiogenic effect of CCK-4 on stress resistant animals is abolished by GB-115. This implies that GB-115 and CCK-4 are acting via the same receptors.

It was shown that, unlike benzodiazepine tranquilizers, GB-115 causes no dependence or tolerance. The compound is almost non-toxic (for peroral administration LD₅₀ > 6000 mg kg⁻¹, p.o.).

Nowadays, GB-115 (2 mg tablets) has passed Phase II of clinical investigations successfully. Data obtained confirmed the experimental findings: GB-115 demonstrates a clear antianxiety action without any side effects.

5. GK-2, A POWERFUL HOMODIMERIC DIPEPTIDE NEUROPROTECTOR

Nerve Growth Factor (NGF) was used as a base for the design of a powerful dipeptide neuroprotector GK-2. Nerve growth factor was discovered in the earlier 1950th by Rita Levi Montalcini as the first protein regulator of the neurons growth and development [50]. NGF neuroprotective activity is a result of its ability to induce the synthesis of antiapoptotic and inhibit the proapoptotic factors [51]. NGF interacts with the specific tyrosine kinase TrkA receptors activating two downstream cascades, MAPK/Erk and PI3K/Akt.

NGF is considered to be a promising agent for the treatment of a wide range of neurodegenerative diseases. However, full-size recombinant NGF can't be used as a therapeutic due to unsatisfactory pharmacokinetic properties and undesirable side effects such as hyperalgesia, cancerogenesis, catastrophic weight loss [50, 52]. This problem may be solved by switching from the full size NGF to its low molecular weight mimetics. However, many attempts to create small neuroprotective molecules on the base of NGF do not lead to dissociation of the main pharmacological and adverse side effects. NGF mimetics were obtained by Longo's laboratory (Stanford University, USA), Saragovis laboratory (McGill University, Canada), Colangelo's group (Milan University, Italy), and Cozzolino's group (Firenze University, Italy); however, a selective therapeutic action has not been attained [53–58].

We hypothesized that separate hairpin structures can be responsible for different neurotrophin effects, because each of them should have its own binding site in the transmembrane tyrosine kinase Trk receptor with the different functions due to activation of separate post-receptor signaling pathways. This gives a reason to expect the receiving of the low molecular weight NGF mimetics free from the adverse effects of the full size protein.

NGF is a homodimer of 118 amino acid residues polypeptide chains. Each of its protomer possess two pairs of antiparallel β -strands, which are linked by three irregular segments exposed out-

ward, which are called loops 1 (28–36 residues), 2 (43–49 residues), and 4 (91–98 residues). Following our hypothesis, we proceeded from the β -turn structure of NGF loop 4 (Asp⁹³-Glu⁹⁴-Lys⁹⁵-Gln⁹⁶), which is the most outward exposed according to X-ray data, and, therefore, it can play the key role in the interaction of NGF with its receptor. When designing we kept the central dipeptide fragment, Glu⁹⁴-Lys⁹⁵, which, considering its geometry, was likely to penetrate most deeply into the receptor binding site and to be most fully recognized by the receptor. The peripheral Asp⁹³ was replaced by its bioisostere, the succinic acid residue, and the Gln⁹⁶ amino acid residue was replaced by the amide group. The goal of these two replacements was to stabilize the β -turn conformation, enhance the compound stability against the splitting by peptidases, and decrease the cost of the synthesis. Since NGF is a homodimer, the two β -turn mimetics were dimerized in a head-to-head fashion via a hexamethylenediamine spacer (Fig. 7).

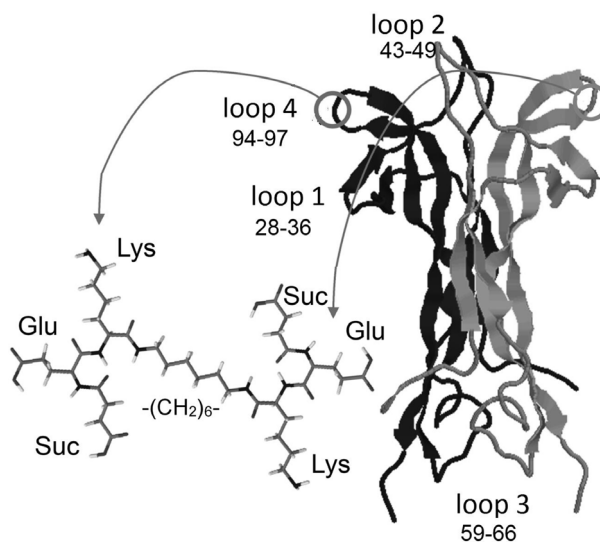


Fig. (7). Design of the homodimeric dipeptide mimetic of loop 4 of the nerve growth factor.

Thus, this approach led us to NGF dipeptide mimetic bis(*N*-monosuccinyl-L-glutamyl-L-lysine) hexamethylenediamide named GK-2 [59, 60].

The experiments in both immortalized HT22 and PC12 cells as well as in the primary rat hippocampal neuronal cells have shown GK-2 in doses of 10⁻⁵–10⁻⁹ M protects neurons from the damaging action of H₂O₂, glutamic acid, 6-hydroxydopamine and MPTP neurotoxins. The magnitude of the GK-2 effect was not inferior to that of native NGF in concentrations of 10⁻⁹ M [61]. In contrast to the full size NGF, GK-2 did not show the differentiating activity in the PC12 cells.

According to Western blot analysis, GK-2 activated TrkA and PI3K/Akt, without affecting the MAPK/Erk signaling [62]. Thus, we obtained a conclusive evidence for the possibility of selective switching on of TrkA signal transduction mechanisms.

It is well-known that the MAPK/Erk signaling participates in the differentiating effect of NGF and hyperalgesia, whereas the neuroprotective effect is carried out mainly through PI3K/Akt pathway.

The neuroprotective activity of GK-2 was confirmed on the animal models of cerebral ischemia [63–65], Parkinson's disease [66], and Alzheimer's disease [67] (Table 1)

The GK-2 was found to be free from the major side effects of NGF. Using the rat tail flick immersion test, it was shown that GK-2 does not increase pain sensitivity [62].

Table 1. Pharmacotherapeutic effects of GK-2 *in vivo*.

Disease	Model	GK-2 Administration Regimen	Effects of GK-2
Cerebral ischemia	Transient ischemic attack in rats caused by bilateral cortical photothrombosis	1 mg/kg (i.p.) subchronically, the first administration 4 h after the surgery	Decrease in the cortical infarct volume by 62%. Complete prevention of retrograde amnesia in active avoidance test
	Ischemic stroke caused by reversible mesencephalic artery occlusion in rats	1 mg/kg (i.p.) subchronically, the first administration 8 h after the surgery	Decrease in the infarct volume by 50% and considerable decrease in the severity of neurological impairment
	Incomplete global ischemia caused by bilateral irreversible carotid artery occlusion in rats	1 mg/kg (i.p.) subchronically, the first administration 4 h after the surgery	Prevention of animal death; restoration of the viability of cortical cells; prevention of the development of cognitive impairment
	Complete global ischemia caused by termination of the general circulation in rats	1 mg/kg (i.p.) within 30 min and then 48 h after revitalization	Decrease in the signs of neurological deficit by 25%; prevention of the damage of the cerebellum Purkinje cells and hippocampal pyramidal neurons in the early (7 days after the surgery) and long term (14 days after the surgery) post-resuscitation period
Parkinson's disease	Haloperidol-induced catalepsy in rats	0.01-5 mg/kg (i.p.) and 5-10 mg/kg (p.o.) 24 h before Haloperidol and 1 mg kg ⁻¹ (i.p.) 45 min after Haloperidol	Decrease in the severity of catalepsy by 80-90%
	MPTP*-induced Parkinsonian syndrome in C57Bl/6 mice	1 mg/kg (i.p.) 24 h before MPTP or subchronically after MPTP	Upon single administration — decrease in the severity of oligokinesia and stiffness; upon subchronical administration after the toxin — complete prevention of the decrease in the step length (stiffness symptom)
	Parkinsonian manifestations in rats induced by unilateral administration of 6-hydroxydopamine into the striatum	1 mg/kg (i.p.) subchronically, the first administration 1 h after the surgery	Complete prevention of apomorphine induced rotations
Alzheimer's disease	Septohippocampal transection in rats	1 mg/kg (i.p.) subchronically, the first administration 2 h after the surgery	Decrease in the degree of the impairment of open field habituation with a therapeutic effect of ~70%
	Cholinergic deficiency caused by long term administration of scopolamine into rats	1 mg/kg (i.p.) subchronically, after scopolamine	Complete prevention of the spatial learning deficit in the Morris water maze
	Bilateral intracerebro-ventricular Streptozotocin administration in rats	0.5 mg/kg (i.p.) subchronically, the first administration 1 h after toxin administration	Prevention of the spatial memory deficiency in the Morris water maze

*MPTP is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

The possible influence of GK-2 on the body weight was evaluated on the rats treated by this agent in the most efficient dose (0.5 mg kg⁻¹) for 14 days. Throughout the whole experiment, no difference was detected between the body weights of animals administered with GK-2 or distilled water [62].

The study of the acute toxicity demonstrated that GK-2 is a moderately toxic compound. The LD₅₀ value for outbred female mice upon a single intravenous administration was found to be 668 mg kg⁻¹, while the same for outbred male mice was 714 mg kg⁻¹. Further monitoring of the mice survived, showed that their condition and behavior became normal after 24 h—they became active, started eating fodder and drinking water. None of them died within 14 days of observation.

A HPLC/MS study of the pharmacokinetics of GK-2 in rats upon i.p. administration demonstrated that the unchanged substance

can be detected in the blood plasma for over 60 min; the substance penetrates through the blood brain barrier.

Thus, we synthesized and pharmacologically investigated a systemically active low molecular weight mimetic of NGF that reproduces its pharmacotherapeutic action *in vivo*, selectively activates the PI3K/Akt signaling pathway, and is free from the major side effects of the full size protein.

CONCLUSION

Thus, we developed new technologies for dipeptide drug design and obtained the dipeptide medicines of various pharmacological groups: Noopept - an effective drug for treatment of cognitive impairment of cerebrovascular or posttraumatic genesis, widely represented in the pharmaceutical market; Dilept, antipsychotic agent with precognitive properties, free from the extrapyramidal side effects (passed the phase II of clinical investigations);

GB-115, a selective anxiolytic with antipanic activity (in the stage of phase III clinical investigations); and GK-2, a neuroprotective drug, potentially useful for the treatment of brain ischemia, Parkinson and Alzheimer's disease prevention (pre-clinical study completed).

Due to easy synthesis, low toxicity, oral availability and similarity to a key fragments of biological active peptides and proteins dipeptide drugs have a high practical potential. The development of the dipeptide trend contributes to medicinal chemistry by providing highly efficient targeted drugs, and to theoretical pharmacology by identifying the minimal active fragments of known neuropeptides, predicting the structures of new endogenous regulatory peptides, and disclosing the previously unknown mechanisms of action of pharmaceutical drugs.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest financial or otherwise.

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