ISSN 0012-4966, Doklady Biological Sciences, 2010, Vol. 431, pp. 79–82. © Pleiades Publishing, Ltd., 2010.
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PHYSIOLOGY

Antiviral Properties of Structural Fragments of the Peptide Selank

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Received October 29, 2009

DOI: 10.1134/S0012496610020031

Widening of the spectrum of antiviral drugs remains an urgent task. Until now, viral diseases, including influenza, herpes, cytomegaloviral infection, and others, are widespread and poorly controlled infections. Therefore, development of new safe antiviral drugs is very important. Peptides constitute one of the most promising classes of physiologically active substances and less studied candidates for creation of these drugs. Peptides have practically no negative side effects because they are endogenous substances. Thus, development of new antiviral drugs on the basis of peptides is an urgent and socially important task.

One of the main problems with the use of natural endogenous peptides for drug development is their multiple functions. We previously reported on the possibility to isolate, from a polyfunctional peptide, a minor amino acid sequence, the so-called pharmacophore, which is responsible for a specific physiological effect [1]. To reveal the minimal amino acid sequence responsible for a specific physiological effect is important for development of new peptides with specific physiological effects.

Another important task in developing peptide drugs is prolongation of their action because they are subjected to intense proteolysis. For prolongation of the effects of newly synthesized peptides, we suggested an approach consisting in protection of unique amino acid sequences responsible for specific effects with natural amino acids or their sequences. Addition of new amino acids or their sequences should not create additional sites for endopeptidases in the pharmacophore. Flanking amino acid sequences should be easily removable by exopeptidases. When necessary, this property may enhance the specific physiological effect or direct its action to a desirable target.

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Among these sequences, the Pro–Gly–Pro tripeptide has proved effective [2]. We studied this C-terminal Pro–Gly–Pro sequence as a candidate protective group when we synthesized peptides that were used as a basis for development of several drugs, including Semax and Selank. Thus, the effect of Semax is approximately 20 times longer as compared to its natural analogue $ACTH_{4–10}$ [3]. Similarly, Selank has a prolonged action as compared to its natural analogue taftsin [4].

Recent experimental studies have shown that glyprolines (GPs) [5], first of all Pro–Gly–Pro, also have specific physiological effects. On the basis of these data, peptides such as Semax and Selank were hypothesized to have hybrid physiological properties. We refer to amino acid sequences that combine the effects of two or more peptides with specific physiological properties as hybrid peptides. A hybrid synthetic peptide specifically combines various physiological effects. These hybrid peptides should express properties of the peptides from which they are composed. On the other hand, each component protects another constituent peptide from the proteases action. For example, Semax consists of the $ACTH_{4–7}$ fragment, which influences cognitive functions, and the Pro-Gly-Pro fragment with neuroprotective properties. The peptide Selank combines neuroprotective and immunotrophic properties of taftsin and the Pro–Gly–Pro neuroprotective action.

Selank has been demonstrated to have antiviral activity. Selank induces the secretion of interferons protecting cell cultures from the cytopathological effects of viruses. Sleank has exhibited antiviral activity against the influenza virus A/Aichi/1/68 strain H_3N_2, experimental herpes infection induced by HSV-2, and experimental viral encephalomyocarditis [6, 7]. Here, we present data on selection of the minimal amino acid sequence or pharmacophore responsible for the antiviral effect of Selank.

On the basis of the above-mentioned concept, we performed our structural and functional studies of peptide sequences and biological studies of their effects in vitro and in vivo, selected from them the

Table 1. Chromatographic and mass-spectrometric analysis of peptide sequences

No.	Peptide	Mol. weight	Chromatographic indices		Mass-spectrometric indices	
			retention time, min	purity, %	$[M + H]^{+}$*	fragmentation of the molecular peak**
1	Met-Glu-His-Phe-Pro-Gly-Pro (Semax)	813	11.68	>98	814	545(100), 517(18), 699(33)
2	Thr-Lys-Pro-Arg-Pro-Gly-Pro (Selank)	751	7.22	>98	752	483(100), 708(98), 655(61)
3	Lys-Pro-Arg-Pro-Gly-Pro	650	11.15	93	651	364(100), 382(99), 426(96)
4	Pro-Arg-Pro-Gly-Pro	522	9.14	89	523	253(100), 425(27), 407(11)
5	Arg-Pro-Gly-Pro	425	6.82	85	426	408(100), 293(54), 254(24)
6	Pro-Gly-Pro	269	5.42	>98	270	173(100), 155(98), 116(75)
7	Thr-Lys	247	1.31	97	248	230(100), 129(78), 84(30)
8	Thr-Lys-Pro	344	4.81	81	345	230(100), 212(17), 129(18)
9	Thr-Lys-Pro-Arg	500	4.65	95	501	457(100), 484(48), 272(51)
10	Thr-Lys-Pro-Gly-Pro	498	6.18	90	499	270(100), 173(11), 155(8)
11	Lys-Pro-Arg	399	4.28	75	400	364(100), 175(54), 382(34)
12	Arg-Pro	271	4.81	75	272	254(100), 175(62), 157(24)
13	Gly-Pro	172	2.40	>98	173	116(100), 127(18), 155(10)
14	Gly-Pro-Gly	229	10.53	>98	230	155(100), 127(17), 213(6)
15	Gly-Pro-Gly-Pro	326	6.02	>98	327	309(100), 173(18), 212(15)
16	Pro-Gly-Pro-Arg	425	6.7	77	426	155(100), 173(98), 116(66)
17	Pro-Gly-Pro-Leu	382	9.89	>98	383	229(100), 286(62), 337(33)

Note: * The molecular peak corresponding to the $[M + H]^{*}$ ion; ** the most intense ions formed during fragmentation of the molecular peak at the energy value of ion hit with 35-eV helium ions.

most promising sequences (in terms their directed antiviral effect), and developed novel drugs.

In accordance with this approach, we synthesized 17 new peptides. The structures of Selank fragments were created after sequential cleavage of N- and C-terminal amino acids and synthesis of various sequences that were homologous to taftsin or Pro–Gly–Pro. Additionally, we examined fragments of Selank with different C-terminal amino acids and various GPs homologous to the Selank sequence. These approaches yielded peptide structures that are presented in Tables 1 and 2. These tables show some features of the peptide synthesized. We present the from the results of high-performance liquid chromatography (HPLC) using a MilliKhrom A-02 liquid-microcolumn chromatograph equipped with a 2 × 75 mm Prontosil 120–5C18aq column. We used eluent A consisting of 0.2 M $LiClO_4$ and 5 mM $HClO_4$ and eluent B, which was methanol. All synthesized peptides were characterized using a ThermoElectron LCQ Advantage MAX mass spectrometer. These chromatographic conditions allowed easily preparing chromatographically pure products. The purity of all synthesized peptides presented in Table 1 was suitable for screening them for antiviral activity.

In Table 1, the structure of the Semax peptide is shown under no. 1. This peptide is already used in clinical practice. Being a hybrid peptide consisting of the $ACTH_{4–7}$ fragment and the Pro–Gly–Pro tripeptide, Semax is homologous to Selank because of the presence of the Pro–Gly–Pro tripeptide. In earlier in vitro and in vivo experiments, we demonstrated antiviral properties of Selank [6, 7]. While the antiviral properties of Selank are related to the Pro–Gly–Pro sequence, Semax and other peptides that contain this sequence must have antiviral properties too.

All biological studies on the antiviral activity of the synthesized peptides were performed in accordance with the methodical recommendations for studies on specific antiviral activities of pharmacological preparations [8].

We estimated the antiviral activities of the peptides at a concentration of 10^{-6} M. We used the peptides as a means for immediate prevention, with the peptides administered simultaneously with a virus infection, as well as a preventive drug administered prior to virus infection, and for treatmen, when the peptides were administered after the virus infection. We performed these studies using the human influenza virus A/Aichi 2/68 strain H_3N_2 infecting a cultured cell strain of dig kidney fibroblasts (MDCK); avian influenza virus

Table 2. The spectrum of peptide antiviral activities in cell cultures

No.	Peptide	Peptide administration protocol		
		prevention	immediate prevention	treatment
1	Met-Glu-His-Phe-Pro-Gly-Pro (Semax)		H_3N_2 HSV-2 CMV	H_5N_1 HSV-2 CMV
2	Thr-Lys-Pro-Arg-Pro-Gly-Pro (Selank)	 HSV-2 CMV	H_3N_2 EVEM HSV-2 CMV	 HSV-2 CMV
3	Lys-Pro-Arg-Pro-Gly-Pro	 HSV-2 CMV	H_3N_2 H_5N_1 HSV-2 CMV	H_5N_1 HSV-2 CMV
4	Pro-Arg-Pro-Gly-Pro	 Influenza B virus HSV-2 CMV	H_3N_2 Influenza B virus EVEM HSV-2 CMV	H_5N_1 Influenza B virus HSV-2 CMV
5	Arg-Pro-Gly-Pro	Influenza B virus HSV-2 CMV	H_3N_2 H_5N_1 Influenza B virus EVEM HSV-2 CMV	H_5N_1 HSV-2 CMV
6	Pro-Gly-Pro	H_3N_2 Influenza B virus HSV-2 CMV	H_5N_1 Influenza B virus EVEM HSV-2 CMV	H_5N_1 HSV-2 CMV
7	Thr-Lys-Pro-Gly-Pro	HSV-2 CMV	H_3N_2 EVEM HSV-2	H_5N_1 HSV-2
8	Thr-Lys-Pro-Arg	H_3N_2 HSV-2	EVEM HSV-2	 HSV-2
9	Thr-Lys-Pro	 HSV-2	EVEM HSV-2	 HSV-2
10	Lys-Pro-Arg	H_3N_2 HSV-2 CMV	EVEM HSV-2	 HSV-2
11	Arg-Pro	H_3N_2 HSV-2 CMV	H_5N_1 EVEM HSV-2	H_3N_2 H_5N_1 HSV-2
12	Thr-Lys	HSV-2 CMV	H_3N_2 EVEM HSV-2	H_5N_1 HSV-2
13	Gly-Pro	H_3N_2 Influenza B virus CMV	H_3N_2 Influenza B virus EVEM	H_3N_2 H_5N_1 HSV-2
14	Gly-Pro-Gly	 Influenza B virus CMV	H_3N_2 Influenza B virus EVEM	 Influenza B virus HSV-2
15	Gly-Pro-Gly-Pro	Influenza B virus CMV	H_3N_2 EVEM	Influenza B virus HSV-2 CMV

Note: H_3N_2, human influenza virus A/Aichi 2/68; H_5N_1, avian influenza virus (2005); Influenza B virus, human influenza virus B/Ohio 01/05; EVEM, mouse encephalomyocarditis virus; HSV-2, type 2 herpes simplex (genital herpes) virus; CMV, cytomegalovirus.

Table 3. Survival of mice infested with encephalomyocarditis virus after peptide treatment

No.	Peptides	Survival, %
1	Met-Glu-His-Phe-Pro-Gly-Pro (Semax)	40
2	Thr-Lys-Pro-Arg-Pro-Gly-Pro (Selank)	20
3	Lys-Pro-Arg-Pro-Gly-Pro	60
4	Pro-Arg-Pro-Gly-Pro	40
5	Arg-Pro-Gly-Pro	100
6	Pro-Gly-Pro	80
7	Thr-Lys-Pro-Gly-Pro	40
8	Thr-Lys-Pro-Arg	20
9	Thr-Lys-Pro	40
10	Lys-Pro-Arg	40
11	Arg-Pro	40
12	Gly-Pro	60
13	Control	20

(H_5N_1) obtained during epizooty among poultry in Novosibirsk oblast in July 2005, which was added to a culture of pig embryonic kidney cells (PEKC); human influenza virus B/Ohio 01/05 added to an MDCK culture; types 1 and 2 herpes simplex viruses (HSVs) added to a culture of African green monkey kidney cells (VERO); cytomegalovirus (CMV) added to an embryonic human lung fibroblast cell culture (EHLF); and mouse encephalomyocarditis virus (MEMC), which was used in vivo.

A higher antiviral activity of the peptides in comparison with the known antiviral drugs was used as a criterion of their antiviral effect. Our in vitro data demonstrated that almost all studied peptides had significant antiviral activities, which depended on the peptide concentration and the time of administration. As a rule, the antiviral effects of these peptides were higher than in the drugs used for comparison. Peptides with significant antiviral effects had proline and glycine on their C-termini. The shortest sequence with a distinct antiviral effect that was comparable with that of comparators was the Gly–Pro peptide. Study of the antiviral properties of the peptides in vivo supported our in vitro data in general. We also studied the survival of mice infected with encephalomyocarditis virus after their treatment with the peptides.

Data from in vivo experiments are presented in Table 3. It has been shown that the shortest Selank fragment with antiviral properties is the Gly–Pro peptide.

Thus, our concept of designing new peptides with desired antiviral properties allowed us to select the shortest amino acid sequence from structural fragments of Selank, which makes it possible to select new peptides suitable for treatment of a number of socially important diseases, such as influenza, herpes, and cytomegalovirus infection.

ACKNOWLEDGMENTS

This study was supported by the Federal Target Program "Research and Developments in Priority Areas of the Scientific and Technological Complex of Russia for the Years 2007-2012" (action 1.2, step VII). Project name, "The Concept for Design of Novel Drugs as Exemplified by Development of Antiviral Peptides and Study of Antiviral Properties of Structural Fragments of the Peptide Selank"; project code, 2008-02-1.2-05-13-018; state contract no. 02.512.11.2245 of July 7, 2008. Chief investigator, Academician N.F. Myasoedov.

REFERENCES

1. Ashmarin, I.P., Samonina, G.E., Lyapina, L.A., et al., *Pathophysiology*, 2005, vol. 11, no. 4, pp. 179–185.
2. Ashmarin, I.P., Kamenskii, A.A., Lyapina, L.A., et al., *Vopr. Biol. Med. i Farm. Khimii*, 2002, no. 1, pp. 24–27.
3. Ponomareva-Stepnaya, M.A., Nezavibat'ko, V.N., Antonova, L.V., et al., *Khim.-Farm. Zh.,* 1984, no. 7, pp. 790–795.
4. Kozlovskaya, M.M., Seredenin, S.B., Kozlovskii, I.I., et al., *Khim.-Farm. Zh.*, 2001, vol. 35, no. 3, pp. 3–5.
5. Martynova, K.V., Andreeva, L.A., Klimova, P.A., et al., *Bioorg. Khim.*, 2009, vol. 35, no. 2, pp. 165–171.
6. RF Patent 2 318 533, *Byull. Izobret.*, 2008, no. 7.
7. RF Patent 2 337 703, *Byull. Izobret.*, 2008, no. 31.
8. Ershov, F.I., Narovlyanskii, A.N., Deryabin, P.G., et al., in *Rukovodstvo po eksperimental'nomu (doklinicheskomu) izucheniyu farmakologicheskikh veshchestv* (A Manual on Experimental (Preclinical) Study of Pharmacological Substances), Moscow, 2005, pp. 532–557.