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Chemical Platform for the Preparation of Synthetic Orally Active Peptidomimetics with Hemoregulating Activity

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A novel chemical platform based on branched piperazine-2,5-dione derivatives (2,5-diketopiperazines) for creating orally available biologically active peptidomimetics has been developed. The platform includes a diketopiperazine scaffold with "built-in" functionally active peptide fragments covalently attached via linkers. The concept was applied to two hemostimulatory drugs, the dipeptide thymogen (GluTrp) and the tripeptide stemokin (IleGluTrp). Preparation of a series of respective derivatives is described. Of the five synthesized analogues, three demonstrated high hemostimulatory activity in vivo on intact mice and on ex vivo irradiated bone marrow cells. Prospects of further development of the concept are discussed.

Peptides regulate a broad range of biochemical reactions in living organisms, and serve as potential prototypes for numerous drug preparations.[1,2] Despite various problems related to the development of peptide drugs, the number of pharmaceuticals based on peptides and peptidomimetics is constantly growing; at present more than 70 peptide preparations have been registered worldwide, including 14 original peptide drug products developed and registered in Russia.[3,4]

Several hundred peptides as potential drug candidates are currently at various stages of preclinical and clinical investigations in many countries. However, the total share of peptides in the global pharmaceutical market (less than 1%) corresponds neither to the major role that peptides play in vital activity of higher organisms, nor to the range of opportunities that multifunctional peptide molecules provide for the creation of new derivatives applicable for regulating virtually any metabolic process.

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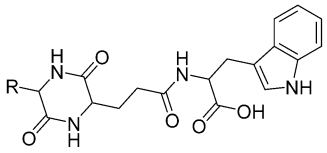
The major disadvantage of this compound class—low stability under non-invasive methods of administration—limits the use of peptide preparations in medical practice.[5] Among the common ways of increasing enzymatic stability of peptide molecules is the introduction of elements with a cyclic structure, which as a rule are resistant to proteolytic degradation.[6–8] In the scientific[7] and patent literature,[9] derivatives of cyclic dipeptides (piperazine-2,5-diones or 2,5-diketopiperazines, DKP) are reported to have been introduced as elements attached to the N or C termini of an active peptide, or incorporate one or two residues of an active peptide in their structure as a new group of protease-resistant peptidomimetics.

This approach was applied in the present work to the dipeptide Glu-Trp (thymogen) **1**, isolated in 1985 from the extract of bovine thymus,[10] and its tripeptide analogue Ile-Glu-Trp (stemokin) **2**. Both preparations are registered in Russia as drugs under the trade names Thymogen® and Stemokin®. Preparations are used in clinical practice in the form of injections.[11] Thymogen® stimulates the differentiation and proliferation of T-lymphocytes and promotes the activity of neutrophils, monocytes, and NK cells, providing a regulatory effect on cellular and humoral immunity.[12] It was also shown that Thymogen® promotes proliferation of damaged bone marrow cells.[13] Stemokin® exhibits an increased affinity for bone marrow cells and is known as a hemostimulatory and immunoadjuvant agent.[14]

In this work we synthesized Glu-Trp-containing diketopiperazines **3**–**7** (Figure 1) and evaluated their hemostimulating activity upon systemic and oral administration. Preliminary communication is given in reference [15]. The selection of the target molecules was based on the following considerations. Analogues **3**–**7** contain a 2,5-diketopiperazine fragment in the N-terminal part of dipeptide **1**. In all cases the Cα-carboxyl group of the glutamic acid residue participates in the formation of



No.	Peptide	R
3	cyclo-[Ala-Glu(Trp)]	$-CH_3$
4	cyclo-[Val-Glu(Trp)]	$-CH(CH_3)_2$
5	cyclo-[Leu-Glu(Trp)]	$-CH_2CH(CH_3)_2$
6	cyclo-[Lys-Glu(Trp)]	$-(CH_2)_4NH_2$
7	cyclo-[Glu(Ile)-Glu(Trp)]	$-(CH_2)_2CO[NHCH(COOH)(CH_3)CH_2CH_3]$

Figure 1. Cyclic analogues of thymogen (**3**–**6**) and stemokin (**7**).

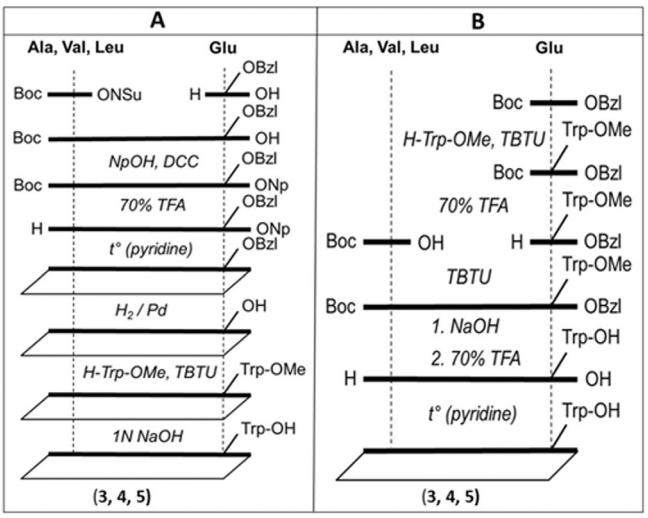


Figure 2. Synthetic schemes for peptides **3**, **4**, and **5**.

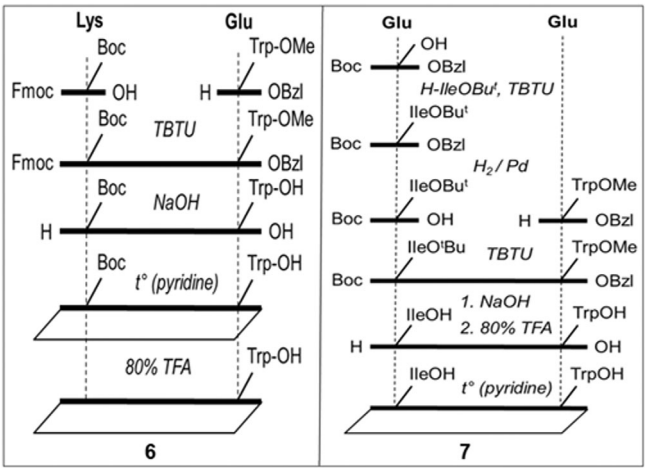


Figure 3. Synthetic schemes for compounds **6** and **7**.

the cyclic fragment, while the amide bond of Glu-Trp, in contrast to thymogen, is formed by the Cγ-carboxyl group. In our earlier work[13, 16–19] it was shown that such structural modification does not impair the biological activity of thymogen and its analogues.[13,17] As the second amino acid participating in the formation of the diketopiperazine cycle, alanine **3**, hydrophobic valine and leucine (**4** and **5**), and lysine with its amino function protonated under standard conditions **6** were used. Peptide **7** is considered as an analogue of stemokin **2**, in which the isoleucine residue is linked to the thymogen-like fragment via the side chain of the second glutamic acid, incorporated into the diketopiperazine cycle. In all cases, residues that form the diketopiperazine core are not only carriers for pharmacophores but also act as active components, built into the structure of the drug candidate.

Synthesis of the substituted diketopiperazines was performed by classical solution-phase methods of peptide chemistry according to synthetic schemes shown in Figures 2 and 3. Standard abbreviations are used as recommended in reference [20]. Two approaches were investigated for the cyclization of linear peptides into DKP derivatives. The first (A) included cyclization of activated esters of protected dipeptides followed by coupling with the third amino acid;[21] the second, B, involved direct cyclization of linear tripeptide precursor by heating in pyridine solution at reflux.[22] Samples of **3**–**5** were obtained according to both approaches. The second one afforded final products with considerably higher overall yields (27, 35, and 34%, versus 14, 16, and 17%) and was chosen for the synthesis of compounds **6** and **7** (Figure 3).

Cyclization kinetics (Figure 4) were followed by analytical HPLC. Experimental details of the syntheses are given in the Supporting Information. An Acquity UPLC system was used (Waters, USA). The column was an Acquity UPLC BEH C_{18} (1.7 μm, 2.1×50 mm). Buffer A consisted of 0.1% formic acid; buffer B was 0.1% formic acid in acetonitrile. Elution was performed by a linear gradient from 5 to 80% buffer B in buffer A for 8 min at a flow rate of 0.5 mL min^{-1}, with detection at λ 220 nm.

Final purification by preparative HPLC [Spherical C_{18} silica gel (Sorbent Technology)] afforded the target products **3**–**7** in 95–

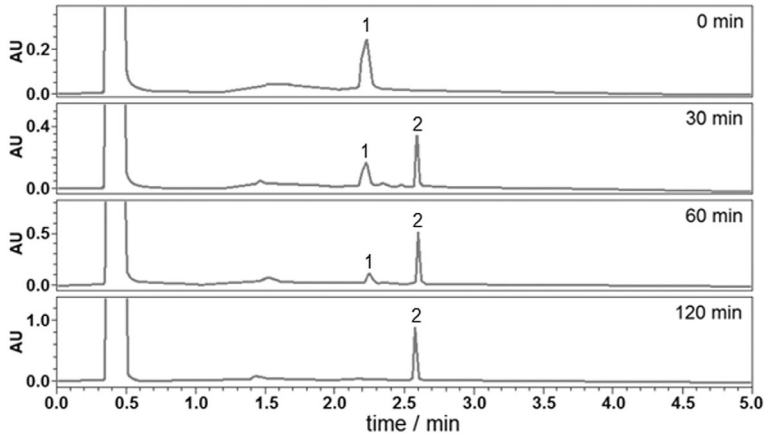


Figure 4. Cyclization kinetics of compound **3** (peak 2) from linear precursor (peak 1).

97% purity. Physicochemical characteristics of peptides **3**–**7** and their linear precursors are provided in the Supporting Information (Table S1). The proteolytic stability of peptide **6** along with a series of its derivatives was demonstrated in one of our earlier works.[7]

Thymogen, stemokin, and other hemoregulatory factors such as colony-, granulocyte-, and granulocyte–macrophage-stimulating factors (CSF, G-CSF, and GM-CSF) affect the proliferation rate and induce differentiation of a variety of blood cells.[23] In our study we evaluated the impact of peptides **3**–**7** on initial studies of hematopoiesis in a test system based on the measurement of colony-forming ability of undifferentiated bone marrow stem cells (CFU-S) on the spleen of sub-lethally irradiated recipients (Figure 5).

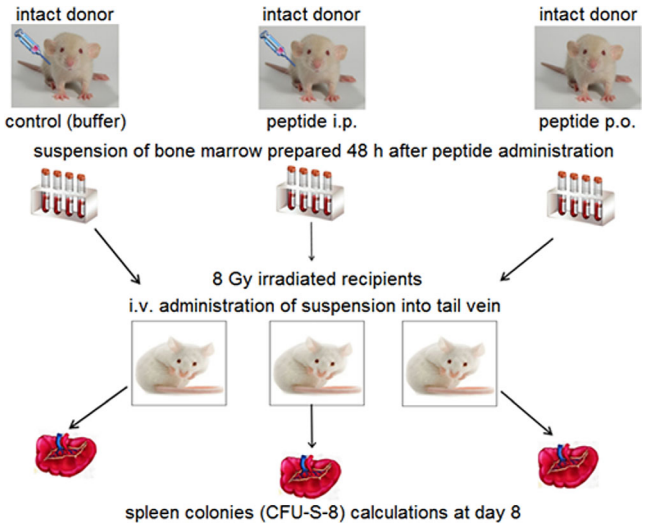


Figure 5. Experimental model for the study of intact bone marrow cell stimulation by peptides.

The method originally introduced in reference [24] was used in our earlier works.[13,19] Suspension of bone marrow cells taken from the femoral bone of intact mice or mice treated by peptide 48 h before the bone marrow extraction was intravenously administrated to sub-lethally irradiated (8 Gy) recipient animals. The immune and hematopoietic systems of such animals are fully destroyed and do not interfere with donor stem cells. The donor bone marrow stem cells form colonies on the surface of the recipient spleen, the number of colonies are measured by microscopy and subjected to analysis. An increase in colony number from cells taken from the peptide-treated animal serves as a measure of the stimulatory action of peptides (Figures 5, 6 and Table S2 in the Supporting Information). As seen from Figure 5 and Table S2 of the Supporting Information, linear prototypes **1** and **2** show practically no effect on the proliferative bone marrow activity of the intact mice both at intraperitoneal injection and at oral application. In contrast, the cyclic analogues **3** and **7** significantly enhance the colony-forming activity of intact bone marrow cells upon both routes of administration. Peptides **4**–**6** were inactive at the standard 100 $\mu g\,kg^{-1}$ intraperitoneal (i.p.) applications.

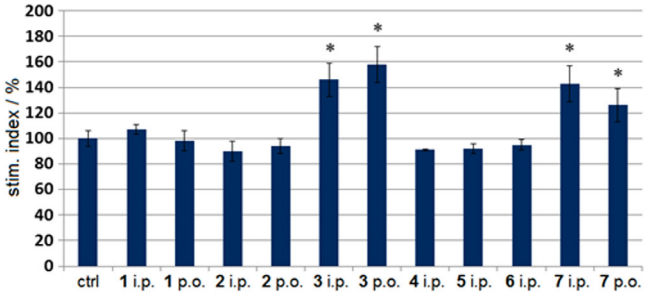


Figure 6. Stimulation of intact bone marrow cell proliferation by peptides **1**–**7**. The ratio of cell colony number on the recipient spleen versus control (in %) is shown; $*p < 0.05$ relative to control.

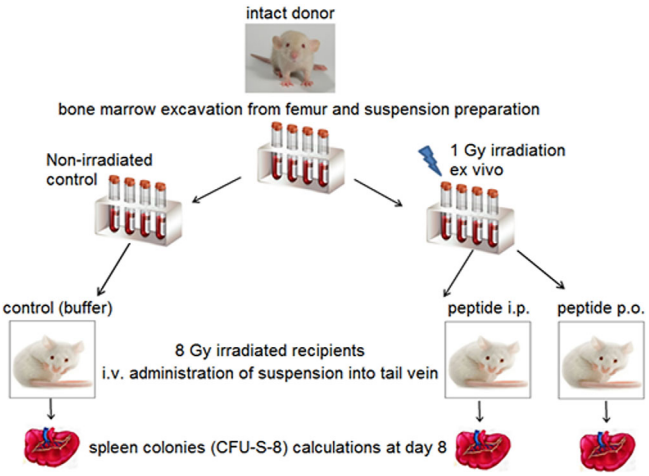


Figure 7. Experimental model for the study of therapeutic action of peptides on damaged bone marrow cells.

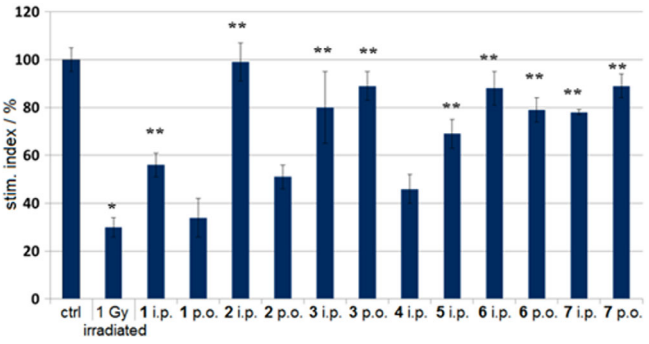


Figure 8. Restoration of damaged bone marrow cell proliferation activity by peptides **1**–**7**. The ratio of cell colony number on the recipient spleen versus control (in %) is shown; $*p < 0.05$ relative to control group, $**p < 0.05$ relative to irradiated group.

In another set of experiments the action of peptides **1**–**7** on damaged bone marrow was studied (Figures 7, 8 and Table S3 in the Supporting Information). As a damaging factor, ex vivo irradiation of the cell suspension from the femoral marrow at a dose of 1 Gy was used. Irradiated and control (intact) suspensions were administered intravenously to 8 Gy irradiated recipients. One hour after transplantation of the irradiated cells the

test peptide or control solution were administered to recipient i.p. or orally at doses of 10 or 100 $\mu g\,kg^{-1}$. Irradiation of bone marrow cells leads to depletion of colony numbers, and peptide treatment is intended to counteract the damage. The results obtained (Figure 8 and Table S3 in the Supporting Information) clearly demonstrate that linear peptides thymogen **1** and stemokin **2**, being highly active under systemic application, show no activity in the post-irradiation cell restoration test upon oral administration. In contrast, cyclic peptides **3**, **6**, and **7** show high activity under both routes of administration, commensurate with the above-mentioned activity of **1** and **2**.

The Leu-containing analogue **5** showed some tendency in activity at systemic application, and the Val-containing analogue **4** showed none. Generally, the behavior of analogues **4**–**6** in the two test systems provides another example of poor predictability of structure–function relationships in complex biological systems.

As a result of the present work, the first orally active hemostimulatory peptides have been prepared on the basis of our original concept. Successful application of the concept paves the way for further application of the Glu-Trp family of peptides in the area of analogues with hemo- and immunosuppressive activity. Another aspect of future work will concern the design and development of orally active chimeric peptide drugs in which the 2,5-diketopiperazine moiety chemically connects the peptide portion with another pharmacophore, not necessarily of peptide nature. These studies are currently underway in our laboratory.

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Keywords: piperazine-2,5-diones · hematopoiesis · peptidomimetics · synthetic peptides

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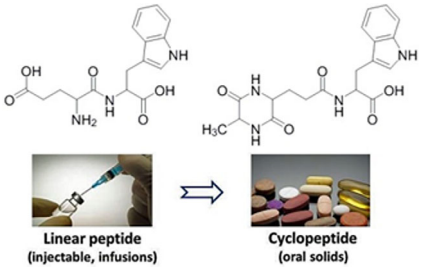
Robust and bioavailable! The low stability of peptide pharmaceuticals in non-invasive administration limits the use of these compounds in medical practice. We developed a platform based on branched piperazine-2,5-diones for creating orally stable peptidomimetics. These derivatives were attached to the N or C termini of an active linear peptide, increasing their resistance against proteolytic activity. As a result, the first orally active hemostimulatory peptides have been prepared.



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