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Synthesis and Analgesic Activity of New Analogues of Tyr-MIF Including Pyrrole Moiety

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Abstract Pain is one of many medical problems of modern society. Together with a number of other diseases such as heart attacks, strokes, tumors, etc. it ranks among the first in manifestation. There are a huge number of medical drugs more or less effective against pain in a practice. Globally, the searching of new molecules with analgesic activity and better selectivity or greater effect at lower doses continues. In addition, some groups trying to improve the properties of known molecules in medical practice as various heterocyclic compounds by modifying one or another of their part. Other groups work on the creation of new mimetics of natural molecules with well established physiological activity. In this global context, here we report the synthesis of two new compounds which are hybrid molecules between the specifically substituted pyrrole (Pyr) and analogues of Tyr-MIF-1 peptide. All investigations on the analgesic activity show better activity at the same dose than natural Tyr-MIF-1 peptide for the analogue Pyr-Tyr-Phe-Leu-Ala-OH. Compound Pyr-Ala-Leu-Phe-Tyr-OH has no better effect comparable to that of the parent peptide. The obtained results clearly show that it is essential that Tyr residue occupies N-terminal position of MIF-1 analogue. The lack of better activity of the analogue Pyr-Ala-Leu-Phe-Tyr-OH reveals that Pyr residue does not influence on the analgesic activity. In addition we found that C-terminal amide function generally presented in natural MIF-1 is not absolutely necessary for activity.

Keywords Opioide peptides · Pyrrole · Pain · Tyr-MIF-1 analogues · Hybride structures · Paal–Knorr reaction

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Introduction

Everyday life does not pass without experiencing some kind of pain. The pain can be caused by various factors. However, in all cases, it represents an unpleasant experience, and in many cases is associated with discomfort and disability. Daily, doctors prescribe large number of painkiller drugs which can belong to different groups—corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), etc. (Dworkin et al. 2003). Since last century studying the properties of various heterocyclic compounds including pyrrole (Pyr), as anti-inflammatory and anti-pain agents is explored strongly (Lainton et al. 1995). It is interesting to note that a number of molecules containing in its structure Pyr heterocycle are approved as drugs with diverse activities in medical practice (Malinka et al. 2000; Amishiro et al. 1999; Bovy et al. 1999; Fumoto et al. 1999; Carson et al. 1997; Artico et al. 2000). On the other hand, some natural molecules including peptides can also perform a variety of functions in the human body. They can be neurotransmitters, neuromodulators, hormones, etc. Tyr-MIF is a tetrapeptide well know from the literature with its opioide activity and

good selectivity according to the μ-receptors (Pan and Kastin 2007; Bocheva et al. 2000; Bocheva and Lazarova 2003). The modification in the structure of some natural opioid peptides, and combining them into hybrid structures with various heterocyclic compounds is investigated as a promising alternative to existing commercial and non-steroidal antiinflammatory and opioid agents.

In this global context, we made a design and synthesized hybrid structures between the Pyr molecule and analogues of Tyr-MIF in order to combine the properties of both types of molecules and to obtain compounds with analgesic activity. Our previous studies [unpublished results] show that the incorporation of single amino acids in a Pyr cycle through the reaction of Paal–Knorr is possible. Our preliminary biological studies indicate that the resulting compounds exhibit a good analgesic activity. Based on these preliminary results, we have made the design of two tetrapeptides, which mimic the structure of the well-known in the literature for its analgesic properties Tyr-MIF. The peptide moiety is a combination of those amino acids which, in our preliminary studies in combination with a Pyr heterocycle showed the highest analgesic activity.

Materials and Methods

Chemical Synthesis

All amino acids and condensation agents are purchased by IRIS Biotech, Germany. All solvents are purchased by Valerus, Bulgaria and they are used without any preliminary treatment. TLC characteristics of aim products were measured on Silica gel 60 F_{254} aluminum sheets, Merck 1.05554 at ambient temperature using chloroform as a mobile phase.

General Procedure for Attachment of Amino Acid to Wang Resin

The Wang resin was pre-swelled with DCM for 1 h and then thoroughly washed with DMF. Fmoc-amino acid (4 eq. to the activated resin) is dissolved in a minimal amount of DCM and diisopropylcarbodiimide (1.65 eq. to the Fmoc-amino acid) is added at 0 °C. The obtained mixture is stirred for 20 min at 0 °C. Further the solvent is removed under vacuum and obtained crude product is dissolved in minimal amount of DMF. The obtained solution is added to the Wang resin in the reactor for SPPS and additional amount of 25 ml of DMF and 0.1 eq. to the resin of dimethylaminopyridin are added. The reaction mixture was stirred for 2 h and after the end of the reaction time it was washed consecutively by 4 × 25 ml of DMF and 4 × 25 ml of DCM. Further the Kaiser test was made after deprotection of small aliquot of the resin by treatment with 20 % piperidine/DMF.

General Procedure for Coupling of Amino Acids by HBTU(TBTU)/DIPEA Method

The Fmoc-amino acid (4 eq. relative to resin loading), HBTU (4 eq. relative to resin loading) and DIPEA (4 eq. relative to resin loading) were dissolved in DMF. The reaction mixture was stirred for 10 min. and added to the peptide-resin solution in DMF. The reaction mixture was left to rest for 2 h. The Kaiser test was made at the end of the reaction time after consecutively washing by 4 × 25 ml of DMF and 4 × 25 ml of DCM.

Preparation of the Peptide-Resin for Cleavage

The obtained peptide-resin was washed by 3× DMF; 3× DCM; 3× i-propanol and 3× Ether to shrink the resin. The peptide-resin was then dried under high vacuum for 4 h over NaOH.

TFA Cleavage and Deprotection of Peptide

The dry peptide-resin was placed in a flask and TFA/TIS/H_2O (95:2,5:2,5) were added for 5 h. At the end of the reaction time the obtained peptide was collected by filtration and the removed resin was washed by 3× TFA and 3× DCM. The obtained filtrates were collected and evaporated to dryness. Final product is recrystallized in cold diethylether.

General Procedure for Paal–Knorr Reaction

Peptide (0.10 mol) was dissolved in glacial acetic acid (50 mL) and to the resulting solution 1,4-dicarbonyl compound (0.10 mol) were added. The obtained solution was refluxed. The reaction development was monitored by TLC. After disappear of starting 1,4-dicarbonyl compound on TLC, the mixture was poured into water. The separated precipitate was filtered off, washed with water, dried, and recrystallized from warm ethanol and wash with 5 % citric acid. The reaction time was varied from 3 to 5 h depending on the starting compounds (TLC control).

LC/MS Analysis

The structure of both aim compounds was proven by LC/MS.

Instrumentation

Analyses were carried out on Q Exactive® hybrid quadrupole-Orbitrap mass spectrometer equipped with

TurboFlow® HPLC system, PAL autosampler and HESI® electrospray ionization module (ThetmoScientific Co, USA). Data acquisition and processing were carried out with XCalibur ® 2.4 software package.

Chromatographic Conditions

Column: synchronis C18, 1.7 μm (50 × 2.1 mm) (ThetmoScientific Co, USA); mobile phase: A = 10 mM ammonium hydrogencarbonate in water, B = A/acetonitrile (10/90, v/v); flow rate: 300 μl/min; gradient: 10 % B for 2 min; 10–90 % B for 15 min; 90 % B for 2 min; 90–10 % B for 1 min and equilibration at 10 % for 3 min. Injection volume: 10.0 μl.

Mass Spectrometry Conditions

Full-scan spectra over the m/z range 150–2000 were acquired in positive ion mode at resolution settings at 70,000. All MS parameters were optimized for sensitivity to the target analytes using the instrument control software program. Q Exactive parameters were: spray voltage 4.0 kV, sheath gas flow rate 35, auxiliary gas flow rate 8, spare gas flow rate 2, capillary temperature 280 °C, probe heater temperature 300 °C and S-lens RF level 50.

Biological Studies

Animals

All the experiments were carried out on male Wistar rats (180–200 g). Animals were housed in groups of 8 per cage and kept under a normal 12 h light/dark cycle and 22 ± 2 °C temperature, with free access to food and water.

Tyr-MIF-1 (was obtained from Sigma) and two newly synthesized compounds Pyr-Tyr-Phe-Leu-Ala-OH (1) and Pyr-Ala-Leu-Phe-Tyr-OH (2) were dissolved in sterile saline solution (0.9 % Na Cl) and injected intraperitoneally (i.p.). All drugs were administrated at a dose 1 mg/kg.

All the experiments were conducted between 9.00 and 12.00 a.m., and according to the ''Principles of laboratory animal care'' 9 (NIH Publication No. 85-23, revised 1985), and the rules of the the Animal Care and Use Committee of the Medical University of Sofia.

Nociceptive Tests

Paw-Pressure Test (Randall–Sellito Test) Description

The changes in the mechanical nociceptive threshold of the rats were measured by analgesimeter (Ugo Basile). Pressure was applied to the hind-paw and the pressure (g) required to elicit nociceptive response (squeak and struggle) was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw (Picture 1).

Hot-Plate (HP) Test Description

The latency of response to pain was measured from the moment of placing an animal on a metal plate (heated to 55 ± 0.5 °C) to the first signs of pain (paw licking, jumping). The cut-off time observed to prevent paw damage was 30 s (Picture 2).

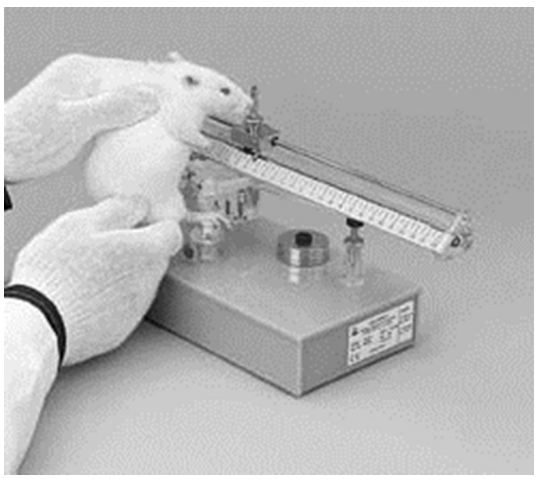
Statistical Analysis

The results for both methods were statistically assessed by one-way analysis of variance ANOVA followed by *t* test comparison. Values are mean ± S.E.M. Values of $p \leq 0.05$ were considered to indicate statistical significance.

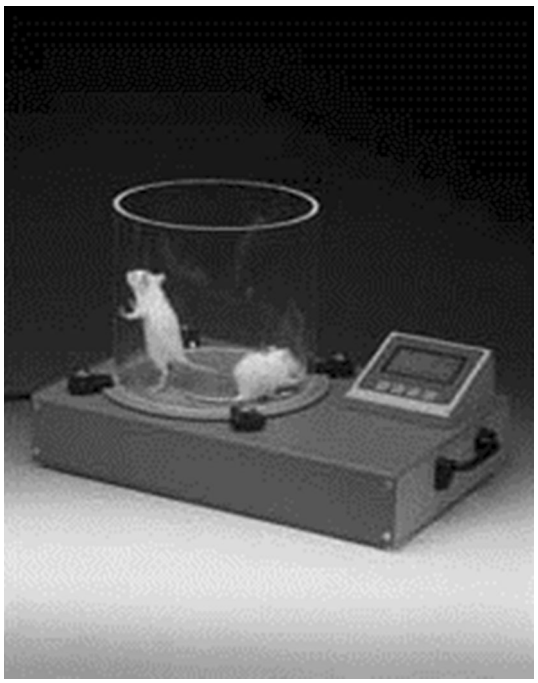
Experimental Part

Synthesis of Pyr-Tyr-Phe-Leu-Ala-OH (1)

H-Tyr-Phe-Leu-Ala-OH (0.10 mol) was dissolved in glacial acetic acid (50 mL) and to the resulting solution 2-[2-(4-chlorophenyl)-2-oxo-ethyl]-3-oxo-butyric acid ethyl ester (0.10 mol) was added. The obtained solution was refluxed for 3–5 h (TLC control). After disappear of starting 2-[2-(4-chlorophenyl)-2-oxo-ethyl]-3-oxo-butyric acid ethyl ester on TLC, the mixture was poured into water. The separated precipitate was filtered off, washed with water, dried, recrystallized from warm ethanol and wash with 5 % citric



Picture 1 Paw pressure (PP) test



Picture 2 Hot plate (HP) test

acid. The obtained product is light brown powder with Rf = 0.58 and yield of 64 %. MS [M + H^+] calculated 758.30824, found 759.31915. t_R (HPLC) = 6.47 min.

Synthesis of Pyr-Ala-Leu-Phe-Tyr-OH (2)

H-Ala-Leu-Phe-Tyr-OH (0.10 mol) was dissolved in glacial acetic acid (50 mL) and to the resulting solution 2-[2-(4-chlorophenyl)-2-oxo-ethyl]-3-oxo-butyric acid ethyl ester (0.10 mol) was added. The obtained solution was refluxed for 3–5 h (TLC control). After disappear of starting 2-[2-(4-chlorophenyl)-2-oxo-ethyl]-3-oxo-butyric acid ethyl ester on TLC, the mixture was poured into water. The separated precipitate was filtered off, washed with water, dried, recrystallized from warm ethanol and wash with 5 % citric acid. The obtained product is yellow powder with Rf = 0.61 and yield of 60 %. MS [M + H^+] calculated 758.30824, found 759.31982. t_R (HPLC) = 7.09 min.

Results and Discussion

Our previous investigation on the hybrid molecules including Pyr heterocycle and single amino acids reveals good activity of some compounds. Based on this study here we made design of two Tyr-MIF analogues H-Tyr-Phe-Leu-Ala-OH and H-Ala-Leu-Phe-Tyr-OH in order to include them in Pyr heterocycle and to study their analgesic activity as well as to reveal some relationships related to the importance of C-terminal amide function for analgesic activity.

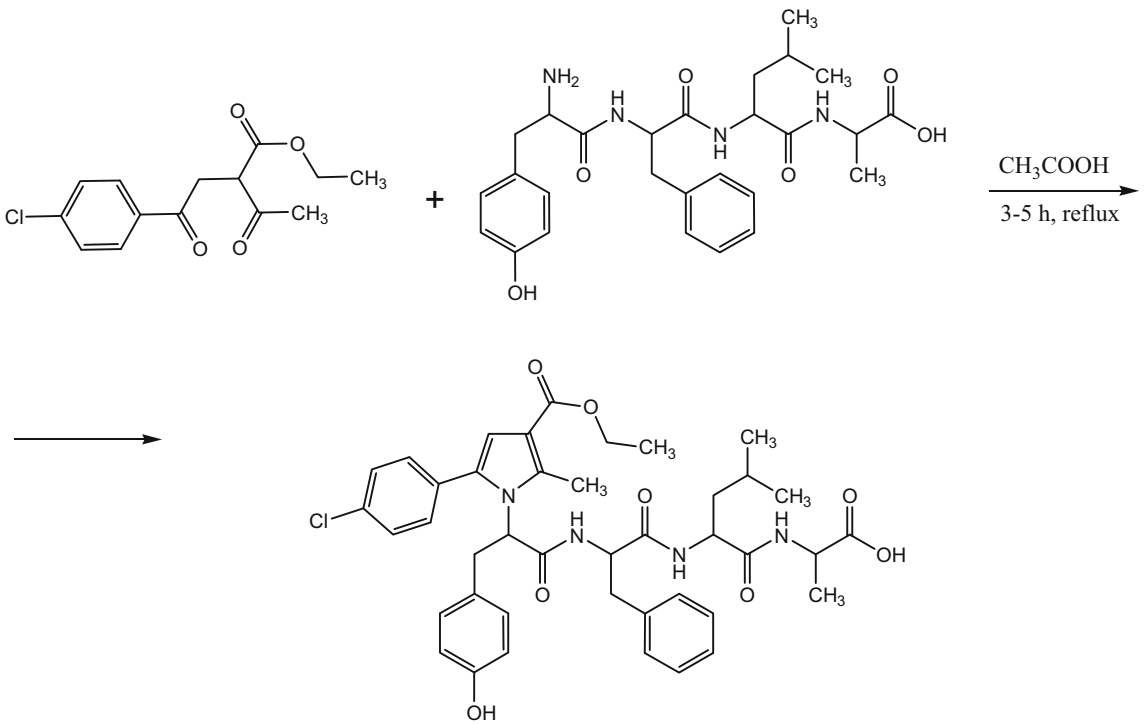


Fig. 1 Synthesis of Pyr-Tyr-Phe-Leu-Ala-OH (1)

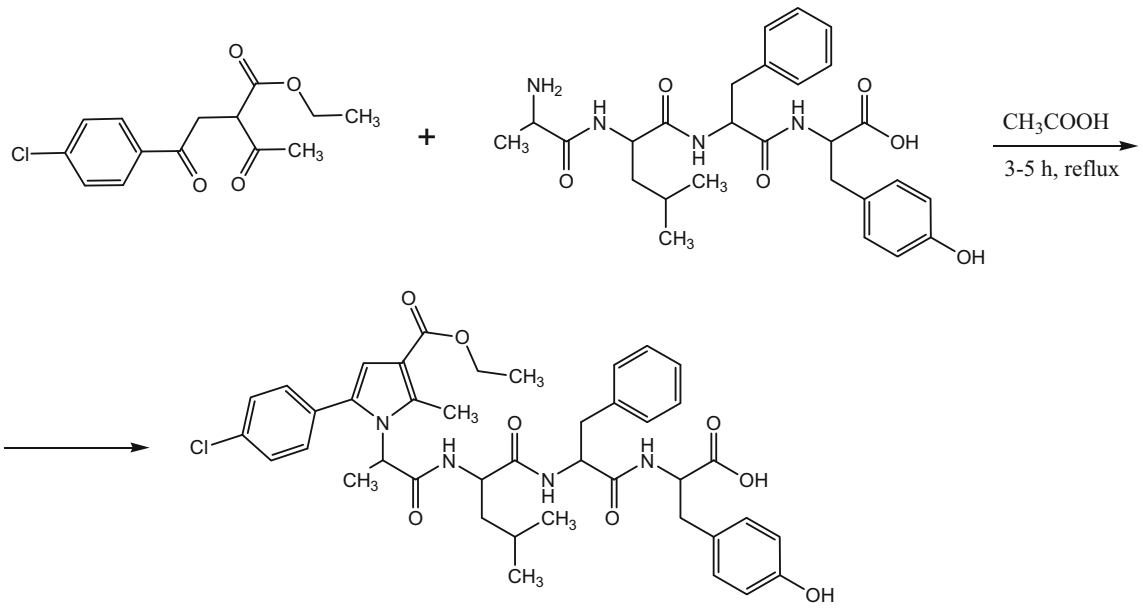


Fig. 2 Synthesis of Pyr-Ala-Leu-Phe-Tyr-OH (2)

The aim peptides were synthesized by standard solid phase peptide synthesis (SPPS) on Wang-polystyrene resin by means of Fmoc/t-Bu strategy.

We met difficulties with the coupling of Fmoc-Leu-OH in both aim peptides and the condensation reaction was repeated three times changing coupling reagents from TBTU, through diisopropylcarbodiimide to HBTU.

The obtained tetrapeptides after deblocking of Fmoc-function of N-terminal amino acid by treatment with 10 % pyperidine/DMF were released of the polymere by treatment with cocktail TFA/TIS/H_2O. During this procedure OH group of Tyr was also deprotected of t-Bu function. Both final tetrapeptide were crystallized in cold diethyl ether and further reaction of Paal–Knorr was used for Pyr heterocycle formation in solution starting from the necessary 1,4-dicarbonyl compound according schemes presented on Figs. 1 and 2.

Structures of two aim hybrid structures are proven by LC/MS.

The investigation started 10 min after i.p. injection of Tyr-MIF-1, Pyr-Tyr-Phe-Leu-Ala-OH (1) and Pyr-Ala-Leu-Phe-Tyr-OH (2) all et a dose 1 mg/kg i.p. Tyr-MIF-1 applied alone significantly increased the pain threshold ($p < 0.001$) in PP test (Fig. 3) and the hot plate latency in HP test (Fig. 4). The analgesic effect was well pronounced and time dependent only in PP test.

The new analogue Pyr-Tyr-Phe-Leu-Ala-OH (1) on the 10th ($p < 0.05$) and 20th ($p < 0.001$) min of investigation significantly increased the pain threshold compared to that of control and Tyr-MIF-1. The analgesic effect is the most significant of the 20th min (Fig. 3).

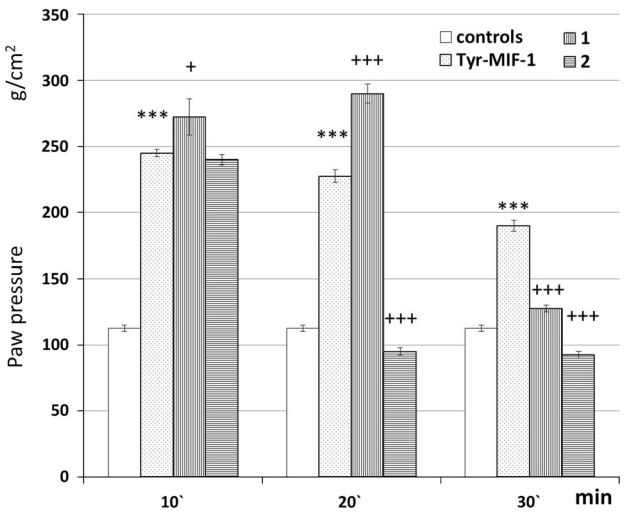


Fig. 3 Effects of Tyr-MIF-1 and the newly synthesized compounds Pyr-Tyr-Phe-Leu-Ala-OH (1) and Pyr-Ala-Leu-Phe-Tyr-OH (2) in a dose 1 mg/kg measured with PP test. Mean values ± S.E.M. are presented. ***$p < 0.001$ relative to control; +++$p < 0.001$ relative to Tyr-MIF-1;+$p < 0.05$ relative to Tyr-MIF-1

The analogue Pyr-Ala-Leu-Phe-Tyr-OH (2) has an analgesic effect comparable to that of the Tyr-MIF-1 peptide only on the 10th min (Fig. 3).

Our results showed that only analogue Pyr-Tyr-Phe-Leu-Ala-OH (1) significantly increased the pain threshold comparable to the control and Tyr-MIF-1.

In HP test Tyr-MIF-1 ($p < 0.001$) and analogue Pyr-Tyr-Phe-Leu-Ala-OH (1) ($p < 0.001$) significantly increased the HP-latency during the whole investigated period, while the

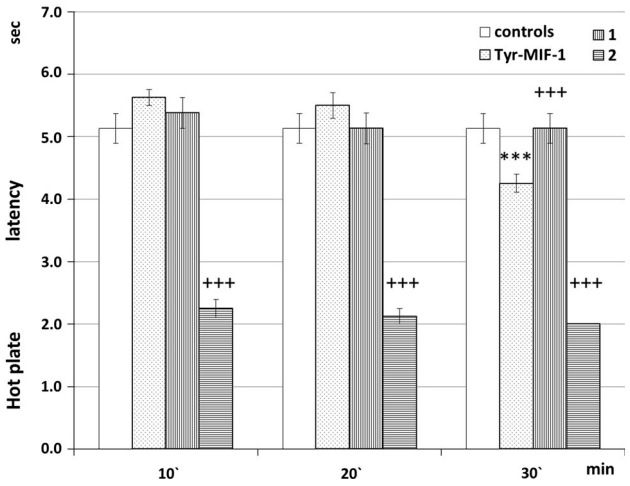


Fig. 4 Effects of Tyr-MIF-1, compounds Pyr-Tyr-Phe-Leu-Ala-OH (1) and Pyr-Ala-Leu-Phe-Tyr-OH (2) measured by hot plate test. Mean values ± S.E.M. are presented. ***$p < 0.001$ relative to control; +++$p < 0.001$ relative to Tyr-MIF-1

hybrid Pyr-Ala-Leu-Phe-Tyr-OH (2) decreased the HP-latency compared to that of control and Tyr-MIF-1.

The obtained results showed that only new analogue Pyr-Tyr-Phe-Leu-Ala-OH (1) significantly increased the HP-latency during the whole investigated period (Fig. 4).

Conclusions

Two peptides analogues of Tyr-MIF-1 were synthesized by SPPS by means of Fmoc/t-Bu strategy. They were further included in their N-terminus in Pyr heterocycle. Studies on the analgesic activity of the two newly synthesized molecules show better activity at the same dose than natural Tyr-MIF-1 peptide for Pyr-Tyr-Phe-Leu-Ala-OH. Compound Pyr-Ala-Leu-Phe-Tyr-OH has no better effect comparable to that of the parent peptide. The obtained results clearly show that it is essential that Tyr residue occupies N-terminal position of MIF-1 analogue. The lack of better activity of the analogue Pyr-Ala-Leu-Phe-Tyr-OH reveals that Pyr moiety does not influence on the analgesic activity. In addition we found that C-terminal amide function generally presented in natural MIF-1 is not absolutely necessary for activity.

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Compliance with Ethical Standards

Conflict of Interest Stanislava P. Vladimirova, Dessislava A. Marinkova, Emilia D. Naydenova and Valentin S. Lozanov declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent Hristina H. Nocheva and Adriana I. Bocheva declare that all the experiments with animals described in this article were conducted between 9.00 and 12.00 a.m., and according to the ''Principles of laboratory animal care'' 9 (NIH publication No. 85-23, revised 1985), and the rules of the the Animal Care and Use Committee of the Medical University of Sofia.

References

Amishiro N, Okamoto A, Murakata C, Tamaoki T, Okabe M, Saito H (1999) Synthesis and antitumor activity of duocarmycin derivatives: Modification segment-A of A-ring pyrrole compounds. J Med Chem 42(15):2946–2960

Artico M, Silvestri R, Pagnozzi E, Bruno B, Novellino E, Greco SM, Ettorre A, Loi AG, Scintu GF, La Colla P (2000) Structure-based design, synthesis, and biological evaluation of novel pyrrolyl aryl sulfones: HIV-1 non-nucleoside reverse transcriptase inhibitors active at nanomolar concentrations. J Med Chem 43(9):1886–1891

Bocheva A, Lazarova M (2003) Involvement of nitric oxide in the nociception of kyotorphin, Tyr-Cav and MIF's analogues in the rats spinal cord. Methods Find Exp Clin Pharmacol 25(2):91–95

Bocheva A, Pajpanova T, Lazarova-Bakarova M, Przewlowska B (2000) Involvement of endogenous NO in nociception of newly synthesized analogues of MIF-1 in acute pain in the rats spinal 26th European peptide symposium, Nice, France, 10–15 Sept 2000. J Pept Sci, 6(Sup):163

Bovy PR, Collins JT, Manning RE (1999) Pharmaceutical compositions for treatment of circulatory disorders using N-substituted (alpha-imidazolyl-toluyl) pyrrole aniotensin II antagonists. US Patent 6,008,368

Carson JR, Carmosin RJ, Pitis PM, Vaught JL, Almond HR, Stables JP, Wolf HH, Swinyard EA, White HS (1997) Aroyl(aminoacyl)pyrroles, a new class of anticonvulsant agents. J Med Chem 40(11):1578–1584

Dworkin RH, Backonja M, Rowbotham MC, Allen RR, Argoff CR, Bennett GJ, Bushnell MC, Farrar JT, Galer BS, Haythornthwaite JA, Hewitt DJ, Loeser JD, Max MB, Saltarelli M, Schmader KE, Stein C, Thompson D, Turk DC, Wallace MS, Watkins LR, Weinstein SM (2003) Advances in neuropathic pain diagnosis, mechanisms, and treatment recommendations. Arch Neurol 60(11):1524–1534

Fumoto Y, Eguchi T, Uno H, Ono N (1999) Synthesis of pyrrolostatin and its analogues. J Org Chem 64(17):6518–6521

Lainton JAH, Huffman JW, Martin BR, Compton DR (1995) 1-Alkyl-3-(1-naphthoyl) pyrroles: a new class of cannabinoid. Tetrahedron Lett 36(9):1401–1404

Malinka W, Sieklucka-Dziuba M, Rajtar G, Rejdak R, Rejdak K, Kleinrok Z (2000) Synthesis of some N-substituted 3,4-pyrroledicarboximides as potential CNS depressive agents. Pharmazie 55(1):9–16

Pan W, Kastin AJ (2007) From MIF-1 to endomorphin: the Tyr-MIF-1 family of peptides, (Review). Peptides 28:2411–2434