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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

MIF-1 and Tyr-MIF-1 analogues containing unnatural amino acids: synthesis, biological activity and docking studies

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Abstract Melanocyte-inhibiting factor (MIF-1) is the first hypothalamic tripeptide which has been demonstrated to act not only in the brain but also in the pituitary. Tyr-MIF-1 acts as an opiate agonist. It binds selectively and with a high affinity to the μ-opioid receptor when compared with the δ-and κ-opioid receptors. A large number of analogues of MIF-1 and Tyr-MIF-1, containing various modifiers in their structures, have been synthesized and their analgesic effect was determined by various in vivo tests. The aim of current study was: (1) to synthesize new MIF-1 and Tyr-MIF-1 analogues containing sulfoarginine (sArg) and norsulfoarginine (NsArg) in the second and third position, respectively; (2) with the help of docking procedures to find the relationship between structure and biological activity of MIF-1 and Tyr-MIF-1 analogues previously synthesized and biologically tested; (3) using found correlation to predict the biological effect of newly synthesized analogues. New analogues of MIF-1 and Tyr-MIF-1 were synthesized using methods of peptide synthesis in solution. Docking was performed with GOLD 5.0 and a correlation between the obtained docking data and the values from in vivo test was found. Some structure–activity relationships were determined. According to the correlation, we made assumptions about the biological effect of sArg and NsArg containing MIF-1 and Tyr-MIF-1. A computational approach could be very useful in the elucidation of the structure–activity relationship and in the design of new analogues with desired biological effect.

Keywords Arginine mimetics · MIF-1 · Tyr-MIF-1 · Nociception · μ-Opioid receptor · Docking · GOLD

Abbreviations

AcOH	Acetic acid
Cav	Canavanine
CNS	Central nervous system
DCM	Dichloromethane
DIPEA	*N,N*-Diisopropylethylamine
DMF	Dimethylformamide
EDAC	*N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide
Et_3N	Triethyl amine
EtOAc	Ethyl acetate
HOBT	Hydroxybenzotriazole
MIF-1	Melanocyte-inhibiting factor
MSH	Melanocyte-stimulating hormone
NCav	Norcanavanine
NMM	*N*-Methylmorpholine
NsArg	Norsulfoarginine
Piv-Cl	Pivaloyl chloride
PP	Paw-pressure nociceptive test
sArg	Sufoarginine
THF	Tetrahydrofuran
Z	Carboxybenzyl

Introduction

Endogenous opioid peptides are an important part of the neuro-hormonal system. They function as hormones, as

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well as neuromodulators. Through these two mechanisms, the endogenous opioid peptides exhibit a wide range of physiological effects, ranging from the relief of pain by the prevention of diarrhoea.

Typical representatives of the endogenous opioid peptides are MIF-1 and Tyr-MIF-1. Melanocyte-inhibiting factor (MIF-1) is the first hypothalamic tripeptide which has been demonstrated to act not only in the brain but also in the pituitary. MIF-1 was first isolated from bovine hypothalamic tissue and has been shown to inhibit the release of melanocyte-stimulating hormone (MSH) (Nair *et al.,* 1971).

Despite sharing three common amino acid residues, MIF-1 is not derived from Tyr-MIF-1 in the blood. It is shown that Tyr-Pro, but not MIF-1, is the primary degradation product of Tyr-MIF-1(Kastin *et al.,* 2001). In other words, Tyr-MIF-1 does not serve as a precursor of MIF-1 (Kastin *et al.,* 1994) (Fig. 1).

Tyr-MIF-1 acts as an opiate agonist. It binds selectively and with a high affinity to the μ-opioid receptor when compared with the δ-and κ-opioid receptors. Tyr-MIF-1 acts as an antagonist of the inhibitory effects of opioids, thus showing some antiopiate effect. These two effects compete, and Tyr-MIF-1 acts as a partial agonist for the μ-opioid receptor. Family of Tyr-MIF-1 peptides exerts more than one central action that lasts longer than their half-life in the blood.

It is known that changes in the structure of the biologically active compounds lead to compounds with a different effect. The peptides are modified in a variety of ways; the most common changes are directed to the substitution of one amino acid by another natural or synthetic, as well as replacement of the peptide bond of the pseudopeptide. All these efforts are aimed at increasing the stability of the corresponding peptide, and also enhancing the desired effect.

Analogues that are the subject of our work and are obtained from a variety of modifications, such as substitution of the tyrosine residue in the first position with a D-isomer of the tyrosine, its methylated, brominated and chlorinated analogues having altered amino acid sequences where the leucine residue is replaced by another natural amino acid or its sulfo-analogue, analogues of peptides coupled with diamines and cinnamic acid. These modifications are shown schematically in Fig. 1.

Materials and methods

Chemistry

All chemicals were of analytical grade. All anhydrous solvents were obtained commercially (Fluka) and used directly. HPLC-grade acetonitrile and MeOH were purchased from Merck. Analytical TLC was performed on Merck silica gel (60F254) plates (0.25 mm) using the following solvent systems: (A) V (C_6H_6):V (CH_3COCH_3):V (CH_3COOH) = 100:50:2; (B) V ($CHCl_3$):V (MeOH):V (CH_3COOH) = 95:5:5; (C) V (CH_3CN):V (H_2O) = 4: 1. Visualization was done with either UV, ninhydrin or a chlorine tolidine reagent. HPLC analyses were performed on Agilent Technologies HP 1100 and Waters 2695 LC instruments, using a Column: Lichrosphere® RP_8 (100 × 4.6 mm); mobile phase: acetonitrile/deionized water 40/60 (v/v). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker WM-250 and Avance DRX-250 (250 and 75 MHz, respectively) spectrometers. Mass spectra were recorded on a Fissons-Triple Quadrupol-ES mass spectrometer.

Amino acids and analogues

Amino acids were obtained from Sigma. Synthesis of unnatural amino acids sulfoarginine and norsulfoarginine was performed according to the previously published reaction procedure (Dzimbova *et al.,* 2011, 2003).

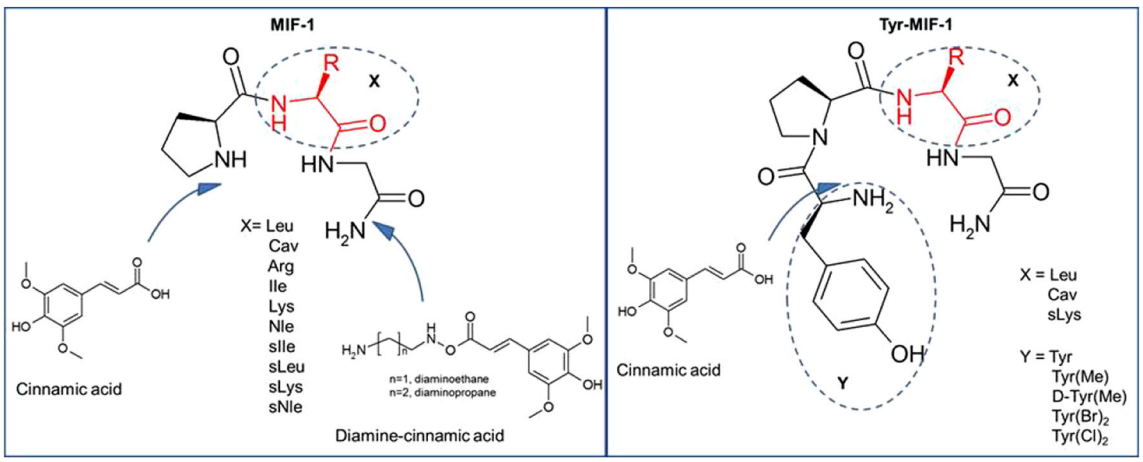

Fig. 1 Modifications in the molecules of MIF-1 and Tyr-MIF-1

Peptide synthesis

The synthesis of many of the analogues of MIF-1 and Tyr-MIF-1 has already been published (Pajpanova *et al.,* 1999; (Pancheva *et al.,* 2005; Spasova *et al.,* 2006; Pancheva *et al.,* 2005). Here, we present the synthesis of the analogues containing sulfoarginine derivatives in their structure.

Synthesis of Z-Pro-sArg-OBzl, benzyl 2-((1-(benzyloxy)-4-(N-(diaminomethylene)sulfamoyl)-1-oxobutan-2-yl)carbamoyl)pyrrolidine-1-carboxylate, ***5a*** *(Z-Pro-NsArg-OBzl, benzyl 2-((1-(benzyloxy)-3-(N-(diaminomethylene)sulfamoyl)-1-oxopropan-2-yl)carbamoyl)pyrrolidine-1-carboxylate,* ***5b***

Z-Pro-OH (0.167 g, 2.6 mM) and DIPEA (0.452 ml, 2.6 mM) were dissolved in CH_2Cl_2 (10 ml). The solution was cooled down to 0 °C, EDAC.HCl (0.498 g, 2.6 mM) was added and after 10 min a solution of HBr*sArg-OBzl (HBr*NsArg-OBzl) (2.6 mM) and Et_3N (0.362 ml, 2.6 mM) in CH_2Cl_2 (10 ml) was added to the reaction mixture. The reaction mixture was stirred for 24 h at room temperature. After the complete reaction, CH_2Cl_2 was evaporated on a rotary vacuum evaporator and the residue was dissolved in EtOAc (20 ml) and was washed subsequently with 5 % $NaHCO_3$ (3 × 10 ml), H_2O, 5 % $NaHSO_4$ (3 × 10 ml), and H_2O. The organic layer was dried over Na_2SO_4, EtOAc was evaporated. The products obtained were used without further purification, 5a (0.27 g, 75 %) and 5b (0.22 g, 62 %). **5a**: MS-ES, *m/z*: 546 [M^+] (545.61); Anal. Calcd. for $C_{25}H_{31}N_5O_7S$: C, 55.03; H, 5.73; N, 12.84; O, 20.53; S, 5.88. Found: C, 54.98; H, 5.71; N, 12.75; O, 20.49; S, 6.07. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.56 (s, 4H, NH_2), 8.03 (H, αNH), 7.47 (m, 4H, Ar), 7.38 (m, 6H, Ar), 5.34 (s, 2H, CH_2), 5.09 (s, 2H, $PhCH_2$), 4.51 (m, H, CH), 4.29 (m, H, CH, Pro), 3.41 (m, 2H, γCH_2), 3.40, 3.30 (m, 2H, CH_2), 2.46 (m, 2H, βCH_2), 1.96, 1.71 (m, 2H, CH_2), 1.64, 1.54 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 171.5, 171.0, 158.5, 154.3, 136.1, 128.9, 127.6, 127.1, 67.1, 66.8, 66.4, 54.8, 53.6, 49.7, 29.4, 24.0, 15.0; **5b**: MS-ES, *m/z*: 532 [M^+] (531.58); Anal. Calcd. for $C_{24}H_{29}N_5O_7S$: C, 54.23; H, 5.50; N, 13.17; O, 21.07; S, 6.03. Found: C, 54.10; H, 5.62; N, 13.11; O, 20.61; S, 6.56. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.53 (s, 4H, NH_2), 8.00 (H, αNH), 7.49 (m, 4H, Ar), 7.41 (m, 6H, Ar), 5.40 (s, 2H, CH_2), 5.05 (s, 2H, $PhCH_2$), 4.4 (m, H, CH), 4.26 (m, H, CH, Pro), 4.1, 3.89 (m, 2H, CH_2), 3.41, 3.33 (m, 2H, CH_2), 1.96, 1.73 (m, 2H, CH_2), 1.61, 1.54 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 171.3, 170.7, 158.0, 154.1, 136.3, 129.2, 127.8, 127.2, 67.5, 66.3, 66.0, 60.5, 49.6, 48.2, 29.9, 24.5.

Synthesis of Z-Pro-sArg-OH, 2-(1-((benzyloxy)carbonyl) pyrrolidine-2-carboxamido)-4-(N-(diaminomethylene) sulfamoyl)butanoic acid, ***6a*** *(Z-Pro-NsArg-OH, 2-(1-((benzyloxy)carbonyl)pyrrolidine-2-carboxamido)-3-(N-(diaminomethylene)sulfamoyl)propanoic acid,* ***6b****)*

Z-Pro-sArg-OBzl (Z-Pro-NsArg-OBzl) (0.4 mM) was dissolved in the mixture of dioxane:water (1:1, 10 ml), and a drop of methanol solution of thymolphthalein was added. 1 N NaOH was added dropwise until the end of the saponification process (blue colour of the solution remained unchanged). Dioxane was evaporated and aqueous solution was extracted with EtOAc (3 × 10 ml), acidified to pH 3 with dry $NaHSO_4$, and was extracted again with EtOAc (3 × 10 ml). Combined organic layers were washed with water, dried over anhydrous Na_2SO_4, and EtOAc was evaporated. Crude products were used without further purification, 6a (0.092 g, 51 %) and 6b (0.081 g, 45 %). **6a**: MS-ES, *m/z*: 456 [M^+] (455.49); Anal. Calcd. for $C_{18}H_{25}N_5O_7S$: C, 47.46; H, 5.53; N, 15.38; O, 24.59; S, 7.04. Found: C, 47.3; H, 5.49; N, 15.16; O, 24.71; S, 7.34. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 11.0 (s, H, OH), 8.50 (s, 4H, NH_2), 8.1 (H, αNH), 7.47 (m, 2H, Ar), 7.33 (m, 3H, Ar), 5.1 (s, 2H, $PhCH_2$), 4.55 (m, H, CH), 4.3 (m, H, CH, Pro), 3.42, 3.28 (m, 2H, CH_2), 3.41 (m, 2H, γCH_2), 2.34 (m, 2H, βCH_2), 1.93, 1.69 (m, 2H, CH_2), 1.66, 1.56 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 174.5, 171.3, 159.5, 154.0, 136.7, 130.2, 128.3, 127.5, 67.1, 66.6, 57.0, 53.4, 49.2, 30.0, 24.3, 14.7; **6b**: MS-ES, *m/z*: 442 [M^+] (441.46); Anal. Calcd. for $C_{17}H_{23}N_5O_7S$: C, 46.25; H, 5.25; N, 15.86; O, 25.37; S, 7.26. Found: C, 46.17; H, 5.29; N, 16.0; O, 25.32; S, 7.22. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 11.05 (s, H, OH), 8.51 (s, 4H, NH_2), 8.07 (H, αNH), 7.43 (m, 2H, Ar), 7.36 (m, 3H, Ar), 5.07 (s, 2H, $PhCH_2$), 4.51 (m, H, CH), 4.27 (m, H, CH, Pro), 4.15, 3.81 (m, 2H, βCH_2), 3.40, 3.33 (m, 2H, CH_2), 1.9, 1.73 (m, 2H, CH_2), 1.65, 1.55 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 174.5, 171.3, 158.1, 154.3, 136.5, 129.3, 128.3, 127.5, 67.0, 66.2, 60.1, 50.4, 49.8, 29.5, 24.1.

Synthesis of Z-Pro-sArg-Gly-NH_2, benzyl 2-((1-((2-amino-2-oxoethyl)amino)-4-(N-(diaminomethylene)sulfamoyl)-1-oxobutan-2-yl)carbamoyl)pyrrolidine-1-carboxylate, ***7a*** *(Z-Pro-NsArg-Gly-NH_2, benzyl 2-((1-((2-amino-2-oxoethyl)amino)-3-(N-(diaminomethylene)sulfamoyl)-1-oxopropan-2-yl)carbamoyl)pyrrolidine-1-carboxylate,* ***7b****)*

A solution of Z-Pro-sArg-OH (Z-Pro-NsArg-OH) (0.2 mM) and NMM (0.023 ml, 0.2 mM) in 5 ml DMF was cooled down to –10 °C and Piv-Cl (0.025 ml, 0.2 mM) was added drop wise. Ten minutes later a solution of HCl.Gly-NH_2 (0.022 g, 0.2 mM) and Et_3N (0.027 ml,

0.2 mM) in 5 ml DMF was added. The reaction mixture was stirred at room temperature for 1 day. After the end of the process 10 ml of water was added and extracted with $CHCl_3$ (3 × 10 ml). The combined organic layers were washed subsequently with 5 % $NaHCO_3$ (3 × 10 ml), 5 % $NaHSO_4$ (3 × 10 ml) and water, the organic phase was dried over anhydrous Na_2SO_4, and the solvent was evaporated. The products were used on the next step without purification, 7a (0.065 g, 65 %) and 7b (0.057 g, 62 %).**7a**: MS-ES, *m/z*: 512 [M^+] (511.55); Anal. Calcd. for $C_{20}H_{29}N_7O_7S$: C, 46.96; H, 5.71; N, 19.17; O, 21.89; S, 6.27. Found: C, 46.72; H, 5.73; N, 19.20; O, 21.84; S, 6.51. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.51 (s, 4H, NH_2), 8.0 (H, αNH), 7.42 (m, 2H, Ar), 7.37 (m, 3H, Ar), 7.16 (s, 2H, NH_2), 5.01 (s, 2H, $PhCH_2$), 4.53 (m, H, CH), 4.32 (m, H, CH, Pro), 4.09 (s, 2H, CH_2), 3.45 (m, 2H, γCH_2), 3.40, 3.25 (m, 2H, CH_2), 2.31 (m, 2H, βCH_2), 1.90, 1.69 (m, 2H, CH_2), 1.63, 1.51 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.5, 172.0, 169.8, 158.2, 154.2, 136.1, 128.7, 127.6, 127.0, 67.3, 66.2, 55.4, 53.1, 49.7, 42.3, 29.4, 24.1, 15.6; **7b**: MS-ES, *m/z*: 498 [M^+] (497.53); Anal. Calcd. for $C_{19}H_{27}N_7O_7S$: C, 45.87; H, 5.47; N, 19.71; O, 22.51; S, 6.44. Found: C, 45.81; H, 5.51; N, 19.68; O, 22.55; S, 6.45. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.53 (s, 4H, NH_2), 8.1 (H, αNH), 8.01 (s, H, NH), 7.42 (m, 2H, Ar), 7.31 (m, 3H, Ar), 7.14 (s, 2H, NH_2), 5.06 (s, 2H, $PhCH_2$), 4.5 (m, H, CH), 4.24 (m, H, CH, Pro), 4.1 (t, 2H, CH_2), 4.04, 3.84 (m, 2H, βCH_2), 3.37, 3.29 (m, 2H, CH_2), 1.91, 1.74 (m, 2H, CH_2), 1.63, 1.53 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.5, 171.4, 169.4, 158.3, 154.0, 136.1, 128.3, 127.3, 126.9, 67.5, 66.4, 61.08, 50.7, 49.4, 42.8, 29.0, 24.3.

Synthesis of Z-Tyr-Pro-OMe, methyl 1-(2-(((benzyloxy) carbonyl)amino)-3-(4-hydroxyphenyl)propanoyl) pyrrolidine-2-carboxylate, ***9***

To the solution of Z-Tyr-OSu (3.1 g, 8.1 mM) in 10 ml DMF, a solution of HCl.Pro-OMe (1.3 g, 8.1 mM) and DiPEA (1.35 ml, 8.1 mM) in 10 ml DMF was added. The reaction mixture was stirred at room temperature for about 4 h and 10 ml of water was added, and extracted with $CHCl_3$ (3 × 10 ml). The combined organic layers were washed subsequently with 5 % $NaHCO_3$ (3 × 10 ml), 5 % $NaHSO_4$ (3 × 10 ml) and water; organic phase was dried over anhydrous Na_2SO_4, and the solvent was evaporated. The crude product was used without further purification, 9 (2.3 g, 67 %). **9**: MS-ES, *m/z*: 427 [M^+] (426.46); Anal. Calcd. for $C_{23}H_{26}N_2O_6$: C, 64.78; H, 6.15; N, 6.57; O, 22.51. Found: C, 64.63; H, 6.18; N, 6.62; O, 22.57. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.04 (s, H, NH), 7.45 (m, 2H, Ar), 7.37 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.70 (m, 2H, Ar), 5.35 (s, H, OH), 5.03 (s, 2H, $PhCH_2$), 4.92 (m, H, CH), 4.27 (m, H, CH, Pro), 3.68 (s, 3H, CH_3), 3.51, 3.41 (m, 2H, CH_2, Pro), 3.44, 3.19 (m, 2H, CH_2), 2.44, 2.19 (m, 2H, CH_2), 2.02, 1.92 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 171.5, 170.2, 155.9, 155.7, 136.3, 130.2, 129.2, 128.9, 127.5, 126.8, 115.8, 66.8, 65.7, 56.6, 51.9, 49.9, 37.6, 28.9, 24.7.

Synthesis of Z-Tyr-Pro-OH, 1-(2-(((benzyloxy)carbonyl) amino)-3-(4-hydroxyphenyl)propanoyl)pyrrolidine-2-carboxylic acid, ***10***

Z-Tyr-Pro-OMe (1.5 g, 3.6 mM) was dissolved in the mixture of dioxane:water (1:1, 20 ml), and a drop of methanol solution of thymolphthalein was added. 1 N NaOH was added dropwise until the end of the saponification process (blue colour of the solution remained unchanged). Dioxane was evaporated and aqueous solution was extracted with EtOAc (3 × 10 ml), acidified to pH 3 with dry $NaHSO_4$, and was extracted again with EtOAc (3 × 10 ml). Combined organic layers were washed with water, dried over anhydrous Na_2SO_4, and EtOAc was evaporated. The crude products were used without further purification, 10 (0.79 g, 55 %). **10**: MS-ES, *m/z*: 413 [M^+] (412.44); Anal. Calcd. for $C_{22}H_{24}N_2O_6$: C, 64.07; H, 5.87; N, 6.79; O, 23.28. Found: C, 64.12; H, 5.89; N, 6.73; O, 23.26. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 11.3 (s, H, OH), 8.01 (s, H, NH), 7.43 (m, 2H, Ar), 7.4 (m, 3H, Ar), 7.15 (m, 2H, Ar), 6.73 (m, 2H, Ar), 5.37 (s, H, OH), 5.07 (s, 2H, $PhCH_2$), 4.9 (m, H, CH), 4.33 (m, H, CH, Pro), 3.55, 3.42 (m, 2H, CH_2, Pro), 3.4, 3.21 (m, 2H, CH_2), 2.33, 2.08 (m, 2H, CH_2), 2.0, 1.91 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 175.9, 170.4, 156.4, 155.1, 136.5, 130.4, 129.7, 129.1, 127.8, 127.0, 115.6, 67.2, 66.6, 56.9, 50.3, 37.9, 28.6, 24.5.

Synthesis of Z-sArg-Gly-NH_2, benzyl (1-((2-amino-2-oxoethyl)amino)-4-(N-(diaminomethylene)sulfamoyl)-1-oxobutan-2-yl)carbamate, ***11a*** *(Z-NsArg-Gly-NH_2, benzyl (1-((2-amino-2-oxoethyl)amino)-3-(N-(diaminomethylene) sulfamoyl)-1-oxopropan-2-yl)carbamate,* ***11b****)*

A solution of Z-sArg-OH (Z-NsArg-OH) (2 mM) and NMM (0.23 ml, 2 mM) in 10 ml DMF was cold down to −10 °C and Piv-Cl (0.25 ml, 2 mM) was added dropwise. 10 min later, a solution of HCl. Gly-NH_2 (0.22 g, 2 mM) and Et_3N (0.27 ml, 2 mM) in 10 ml DMF was added. The reaction mixture was stirred at room temperature for 24 h. 20 ml water was added and the mixture was extracted with $CHCl_3$ (3 × 20 ml). The combined organic layers were washed consequently with 5 % $NaHCO_3$ (3 × 20 ml), 5 % $NaHSO_4$ (3 × 20 ml) and water; the organic phase was dried over anhydrous Na_2SO_4, and the solvent was evaporated. The products obtained 11a (0.5 g, 61 %) and 11b (0.47 g, 59 %) were used on the next step without further purification. **11a**: MS-ES, *m/z*: 415 [M^+] (414.44); Anal.

Calcd. for $C_{15}H_{22}N_6O_6S$: C, 43.47; H, 5.35; N, 20.28; O, 23.16; S, 7.74. Found: C, 43.51; H, 5.31; N, 20.22; O, 23.19; S, 7.77. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.58 (s, 4H, NH_2), 8.05 (s, H, αNH), 8.0 (s, H, NH), 7.44 (m, 2H, Ar), 7.4 (m, 3H, Ar), 7.19 (s, 2H, NH_2), 5.11 (s, 2H, $PhCH_2$), 4.57 (m, H, CH), 4.12 (s, 2H, CH_2), 3.41 (m, 2H, γCH_2), 2.37 (m, 2H, βCH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.8, 169.9, 158.4, 155.9, 136.4, 128.5, 127.8, 127.4, 66.7, 56.7, 53.4, 42.5, 15.5; **11b**: MS-ES, *m/z*: 401 [M^+] (400.41); Anal. Calcd. for $C_{14}H_{20}N_6O_6S$: C, 41.99; H, 5.03; N, 20.99; O, 23.97; S, 8.01. Found: C, 41.95; H, 5.05; N, 21.01; O, 23.92; S, 8.07. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.55 (s, 4H, NH_2), 8.11 (H, αNH), 8.05 (s, H, NH), 7.5 (m, 2H, Ar), 7.37 (m, 3H, Ar), 7.18 (s, 2H, NH_2), 5.12 (s, 2H, $PhCH_2$), 4.52 (m, H, CH), 4.15 (t, 2H, CH_2), 4.01, 3.82 (m, 2H, βCH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.1, 169.6, 158.7, 156.3, 136.5, 129.3, 127.7, 126.8, 66.9, 61.04, 52.3, 42.1.

Synthesis of Z-Tyr-Pro-sArg-Gly-NH_2, benzyl (1-(2-((1-((2-amino-2-oxoethyl)amino)-4-(N-(diaminomethylene) sulfamoyl)-1-oxobutan-2-yl)carbamoyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)carbamate, ***13a*** *(Z-Tyr-Pro-NsArg-Gly-NH_2, benzyl (1-(2-((1-((2-amino-2-oxoethyl)amino)-3-(N-(diaminomethylene)sulfamoyl)-1-oxopropan-2-yl)carbamoyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)carbamate,* ***13b****)*

A solution of Z-Tyr-Pro-OH (0.5 g, 1.2 mM) and NMM (0.13 ml, 1.2 mM) in 10 ml DMF was cooled down to −10 °C and Piv-Cl (0.15 ml, 1.2 mM) was added drop wise. 10 min later a solution of HBr.sArg-Gly-NH_2 (HBr.NsArg-Gly-NH_2) (1.2 mM) and Et_3N (0.17 ml, 1.2 mM) in 10 ml DMF was added. The reaction mixture was stirred at room temperature for 24 h. 20 ml water was added and the mixture was extracted with $CHCl_3$ (3 × 20 ml). The organic layer was washed consequently with 5 % $NaHCO_3$ (3 × 20 ml), 5 % $NaHSO_4$ (3 × 20 ml), and water, and then dried over anhydrous Na_2SO_4, and the solvent was evaporated. The products obtained 13a (0.38 g, 48 %) and 13b (0.41 g, 51 %) were used without further purification. **13a**: MS-ES, *m/z*: 675 [M^+] (674.73); Anal. Calcd. for $C_{29}H_{38}N_8O_9S$: C, 51.62; H, 5.68; N, 16.61; O, 21.34; S, 4.75. Found: C, 51.57; H, 5.71; N, 16.62; O, 21.28; S, 4.82. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.54(s, 4H, NH_2), 8.04 (s, 3H, αNH), 7.48 (m, 2H, Ar), 7.39 (m, 3H, Ar), 7.18 (s, 2H, NH_2), 7.12 (m, 2H, Ar), 6.71 (m, 2H, Ar), 5.32 (s, H, OH), 5.11 (s, 2H, $PhCH_2$), 4.92 (t, H, CH), 4.55 (t, H, CH), 4.40 (m, H, CH, Pro), 4.12 (s, 2H, CH_2), 3.51, 3.41 (m, 2H, CH_2), 3.44, 3.19 (m, 2H, CH_2), 3.42(m, 2H, γCH_2), 2.34 (m, 2H, βCH_2), 2.31, 2.09 (m, 2H, CH_2), 2.0, 1.90 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.9, 171.04, 170.5, 169.4, 158.7, 155.9, 155.4, 136.5, 130.1, 129.5, 128.4, 127.2, 126.7, 115.5, 67.9, 66.5, 55.9, 55.1, 53.6, 49.1, 37.4, 29.5, 24.6, 15.3; **13b**: MS-ES, *m/z*: 661 [M^+] (660.70); Anal. Calcd. for $C_{28}H_{36}N_8O_9S$: C, 50.90; H, 5.49; N, 16.96; O, 21.79; S, 4.85. Found: C, 50.85; H, 5.52; N, 16.93; O, 21.78; S, 4.95. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.57 (s, 4H, NH_2), 8.07 (s, 3H, αNH), 7.45 (m, 2H, Ar), 7.34 (m, 3H, Ar), 7.22 (m, 2H, Ar), 7.12 (s, 2H, NH_2), 6.74 (m, 2 J, Ar), 5.30 (s, H, OH), 5.06 (s, 2H, $PhCH_2$), 4.99 (m, H, CH), 4.57 (m, H, CH), 4.44 (m, H, CH), 4.1 (m, 2H, CH_2), 4.05, 3.77 (m, 2H, βCH_2), 3.54, 3.47 (m, 2H, CH_2), 3.41, 3.2 (m, 2H, CH_2), 2.3, 2.1 (m, 2H, CH_2), 2.01, 1.88 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.1, 171.7, 170.6, 169.7, 158.5, 156.2, 155.4, 136.3, 130.4, 129.1, 128.7, 127.9, 127.3, 67.9, 66.5, 61.07, 56.7, 50.8, 49.3, 42.5, 37.1, 29.1, 24.0.

*Synthesis of Pro-sArg-Gly-NH_2, N-(1-((2-amino-2-oxoethyl)amino)-4-(N-(diaminomethylene)sulfamoyl)-1-oxobutan-2-yl)pyrrolidine-2-carboxamide, (****8a****), Pro-NsArg-Gly-NH_2, N-(1-((2-amino-2-oxoethyl)amino)-3-(N-(diaminomethylene)sulfamoyl)-1-oxopropan-2-yl) pyrrolidine-2-carboxamide, (****8b****), sArg-Gly-NH_2, 2-amino-N-(2-amino-2-oxoethyl)-4-(N-(diaminomethylene) sulfamoyl)butanamide, (****12a****), NsArg-Gly-NH_2, 2-amino-N-(2-amino-2-oxoethyl)-3-(N-(diaminomethylene) sulfamoyl)propanamide, (****12b****), Tyr-Pro-sArg-Gly-NH_2, N-(1-((2-amino-2-oxoethyl)amino)-4-(N-(diaminomethylene)sulfamoyl)-1-oxobutan-2-yl)-1-(2-amino-3-(4-hydroxyphenyl)propanoyl)pyrrolidine-2-carboxamide, (****14a****), and Tyr-Pro-NsArg-Gly-NH_2, N-(1-((2-amino-2-oxoethyl)amino)-3-(N-(diaminomethylene) sulfamoyl)-1-oxopropan-2-yl)-1-(2-amino-3-(4-hydroxyphenyl)propanoyl)pyrrolidine-2-carboxamide, (****14b****)*

The N-Protected peptide analogue (0.1 mM) was dissolved in 1 ml AcOH, and HBr/AcOH (0.5 ml) was added. A deprotection was carried out for 1 h at room temperature. The solvent was removed and the crude product was treated three times with MeOH (3 × 20 ml), which was also evaporated. The HBr-salts of the kyotorphin analogues obtained were transformed to free bases with Et_3N in CH_2Cl_2. The CH_2Cl_2 was evaporated. The final peptides were obtained after column purification (Silicagel 60, CH_3CN: H_2O, 4:1). **8a** (0.03 g, 78 %): MS-ES, *m/z*: 378 [M^+] (377.42); Anal. Calcd. for $C_{12}H_{23}N_7O_5S$: C, 38.19; H, 6.14; N, 25.98; O, 21.20; S, 8.50. Found: C, 38.22; H, 6.10; N, 25.95; O, 21.21; S, 8.52. ^{1}H-NMR ($CDCl_3$) *δ*/ppm: 8.50(s, 4H, NH_2), 8.1 (s, 2H, αNH), 7.10 (s, 2H, NH_2), 4.50 (t, H, CH), 4.12 (s, 2H, CH_2), 3.69 (m, H, CH), 3.4(m, 2H, γCH_2), 2.31 (m, 2H, βCH_2), 2.80, 2.70 (m, 2H, CH_2), 2.04 (m, H, NH), 1.95, 1.73 (m, 2H, CH_2), 1.64, 1.52 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) *δ*/ppm: 172.5, 171.07, 170.4, 158.8, 62.7, 55.8, 53.2, 45.6, 42.5, 30.8, 24.0, 15.1; **8b** (0.028 g, 77 %): MS-ES, *m/z*: 364 [M^+] (363.39); Anal. Calcd. for $C_{11}H_{21}N_7O_5S$: C, 36.36; H, 5.82; N, 26.98; O,

22.01; S, 8.82. Found: C, 36.40; H, 5.81; N, 26.94; O, 22.10; S, 8.75. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.55 (s, 4H, NH_2), 8.11 (s, 2H, αNH), 7. 2 (s, 2H, NH_2), 4.51 (m, H, CH), 4.14 (m, 2H, CH_2), 4.02, 3.72 (m, 2H, βCH_2), 3.71 (m, H, CH), 2.81, 2.75 (m, 2H, CH_2), 1.95, 1.77 (m, 2H, CH_2), 1.6, 1.49 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 172.06, 171.2, 169.2, 159.0, 62.9, 61.06, 51.3, 45.3, 42.0, 31.1, 25.0; **12a** (0.026 g, 92 %): MS-ES, *m/z*: 281 [M^+] (280.30); Anal. Calcd. for $C_7H_{16}N_6O_4S$: C, 29.99; H, 5.75; N, 29.98; O, 22.83; S, 11.44. Found: C, 30.01; H, 5.70; N, 29.92; O, 22.88; S, 11.49. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.6 (s, 4H, NH_2), 8.01 (s, H, αNH), 7.21 (s, 2H, NH_2), 5.11 (s, 2H, NH_2), 4.08 (s, 2H, CH_2), 3.45 (m, 2H, γCH_2), 3.37 (m, H, CH), 2.3 (m, 2H, βCH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 171.3, 169.2, 160.4, 53.1, 51.9, 42.0, 18.3; **12b** (0.023 g, 88 %): MS-ES, *m/z*: 267 [M^+] (266.28); Anal. Calcd. for $C_6H_{14}N_6O_4S$: C, 27.06; H, 5.30; N, 31.56; O, 24.03; S, 12.04. Found: C, 27.10; H, 5.25; N, 31.58; O, 24.00; S, 12.07. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.65 (s, 4H, NH_2), 8.0 (s, H, NH), 7.23 (s, 2H, NH_2), 5.15 (s, 2H, NH_2), 4.11 (t, 2H, CH_2), 4.0, 3.8 (m, 2H, βCH_2), 3.6 (m, H, CH); ^{13}C-NMR ($CDCl_3$) δ/ppm: 172.7, 169.4, 158.1, 63.4, 47.3, 42.16; **14a** (0.05 g, 92 %): MS-ES, *m/z*: 541 [M^+] (540.59); Anal. Calcd. for $C_{21}H_{32}N_8O_7S$: C, 46.66; H, 5.97; N, 20.73; O, 20.72; S, 5.93. Found: C, 46.70; H, 5.95; N, 20.76; O, 20.70; S, 5.89. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.51 (s, 4H, NH_2), 8.07 (s, 2H, αNH), 7.26 (s, 2H, NH_2), 7.0 (m, 2H, Ar), 6.71 (m, 2H, Ar), 5.39 (s, H, OH), 5.07 (s, 2H, NH_2), 4.52 (t, H, CH), 4.47 (m, H, CH, Pro), 4.19 (s, 2H, CH_2), 3.96 (m, H, CH), 3.54, 3.46 (m, 2H, CH_2), 3.47, 3.25 (m, 2H, CH_2), 3.4 (m, 2H, γCH_2), 2.37 (m, 2H, βCH_2), 2.3, 2.1 (m, 2H, CH_2), 1.97, 1.89 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 173.3, 172.1, 170.9, 169.9, 160.5, 155.4, 130.5, 129.1, 116.2, 68.3, 55.7, 53.0, 51.7, 49.7, 42.8, 39.4, 20.1, 24.0, 15.1; **14b** (0.048 g, 94 %): MS-ES, *m/z*: 527 [M^+] (526.57); Anal. Calcd. for $C_{20}H_{30}N_8O_7S$: C, 45.62; H, 5.74; N, 21.28; O, 21.27; S, 6.09. Found: C, 45.65; H, 5.76; N, 21.31; O, 21.25; S, 6.03. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.51 (s, 4H, NH_2), 8.1 (s, 2H, αNH), 7.19 (s, 2H, NH_2), 7.02 (m, 2H, Ar), 6.67 (m, 2H, Ar), 5.4 (s, H, OH), 5.05 (s, 2H, NH_2), 4.6 (m, H, CH), 4.51 (m, H, CH), 4.09, 3.8 (m, 2H, βCH_2), 4.13 (m, 2H, CH_2), 3.87 (m, H, CH), 3.57, 3.41 (m, 2H, CH_2), 3.31, 3.06 (m, 2H, CH_2), 2.29, 2.07 (m, 2H, CH_2), 2.0, 1.81 (m, 2H, CH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 173.8, 172.4, 171.1, 168.9, 159.3, 156.0, 129.8, 128.7, 116.5, 68.0, 61.0, 51.8, 50.5, 49.0, 38.8, 29.9, 23.9.

Activity Assays

Animals

Male Wistar rats (180–200 g) were used and kept under normal conditions at ambient room temperature (22 °C). The experimental procedures were carried out in accordance with the institutional guidance and general recommendations on the use of animals for scientific purposes.

Paw-pressure test (PP)

Changes in the mechanical nociceptive threshold of rats were measured using an analgesimeter (Ugo Basile) (Randall and Selitto, 1957). The pressure was applied to the dorsal surface of rat hind paw, and the pressure (g) required eliciting nociceptive responses such as squeak, and struggle was taken as mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent paw damage.

Data analysis

The results were statistically assessed by an analysis of variance (ANOVA). All results are expressed as mean ± SEM. One-way analysis of variance was used to verify the statistical significance at $P < 0.05$ between the treated and control groups.

Computational tools

In order to perform computational studies a different software was used in the present work: crystal structure of the μ-opioid receptor was obtained from RCSB (PDB id: 4dkl, http://www.rcsb.org/pdb/home/home.do); ligand preparation was done with Avogadro (an open-source molecular builder and visualization tool—Version 1.0.3, http://avogadro.openmolecules.net/); docking studies were performed using GOLD 5.1 (Genetic Optimization for Ligand Docking, (Jones *et al.,* 1997), run on Scientific LINUX 5.5 operating system; for generation figures, Molegro Molecular Viewer (http://molegro.com/index.php) was used.

Results and discussion

Among the numerous presently known biologically active peptides, neuropeptide hormones are a quite wide group that affects the central nervous system (CNS). Immediately after decryption of the primary structure of these compounds were synthesized tens and sometimes hundreds of fragments and derivatives thereof in the search for more active analogues and to detect the relationship chemical structure–biological activity. Considering the previously known data in the literature for embedding canavanine in model peptides, on the one hand, and the structural similarity of NsArg and NCav with arginine, we decided to investigate the effect of substitution of the amino acid leucine with NsArg derivatives.

The sulfo-analogues of the amino acids were synthesized using original method (Pancheva *et al.,* 2012). Peptide analogues, themselves, have been synthesized during the years using various methods of peptide synthesis: in solution and solid-phase peptide synthesis (Pajpanova *et al.,* 1999; Pancheva *et al.,* 2004).

The synthesized peptide analogues were subjected to various nociceptive tests. For instance, in earlier studies the antinociceptive effects were evaluated using tail flick (TF) and hot plate (HP) tests. It was found that substitution of Leu in position **2** of MIF-molecule by Cav and sLys increased the pain threshold. The analgesic effect with Cav-substitution was highest, whereas parent MIF-1 showed only a min increase of pain threshold. Further we investigate the analgesic effects of the synthesized analogues containing non-protein amino acids Cav and sLys in position 2, modified in N-terminus with sinapic acid (SA) and/or modified in C-terminus with biogenic amines and SA. All of the tested analogues exhibited naloxone-reversible significant analgesic activity (Pajpanova, 2009).

For the purpose of our current work, from nociceptive tests, this by applying mechanical irritation—Paw-pressure test was selected for comparison of their action. After applying the method to all 22 peptides presented in the Table 1, it was found that in contrast to the native MIF-1, having a weak analgesic effect, the analogues (with the exception of the MIF-1 analogues, modified in a second position with arginine, lysine and canavanine) have pronounced antinociceptive effect at 15th min of the experiment, which is antagonized by Naloxone (1 mg/kg, *i.p.*). Values obtained by this method at the 15th min of the experiment are presented in Table 1 and Fig. 2.

From the values obtained from in vivo experiments, it can be concluded that a modified structure of MIF-1and Tyr-MIF-1with the non-protein amino acids as well as analogues coupled with cinnamic acid exhibited analgesic effect at the 15th min of the experiment. This effect, in most cases, is significantly stronger than the effect of MIF-1, and in some cases even stronger than the effect of Tyr-MIF-1. Brominated analogue of Tyr-MIF-1 and the analogue MIF-1 modified at position 2 with norsulfoleucine exhibit stronger analgesic effect than Tyr-MIF- 1. The effects of other analogues of the MIF-1and Tyr-MIF-1 are in the range of 101–246 g/cm^2.

Besides analogues whose biological effect is already known, in our work, we conducted analysis of newly synthesized analogues of MIF-1 and Tyr-MIF-1 with unknown biological effects. These analogues contain in their structure sulfoanalogues of natural amino acid arginine at the position 2 and 3, respectively. The structures of the synthesized new analogues are presented in Fig. 3. Once we find correlation between the structure and the known biological activity, an attempt will be made to predict the biological activity of the newly synthesized compounds. This predictive activity will be checked by the method described above; and it is expected to confirm the found relationship.

Table 1 Date for the ligands from in vivo test and docking studies

No.	Ligand	Paw-pressure test, 15 min/g/cm^2	Values of ChemScore function
1	[D-Tyr(Me)-MIF-1]	178	20.63
2	[MIF-1]	101	14.21
3	[MIF-1 Arg^2]	110	5.43
4	[MIF-1 Cav^2]	130	3.27
5	[MIF-1 Eda SA]	206	21.09
6	[MIF-1 Ile^2]	223.33	14.92
7	[MIF-1 Lys^2]	128	10.76
8	[MIF-1 NLe^2]	220	14.02
9	[MIF-1 Pda SA]	230	16.90
10	[MIF-1 sIle^2]	140	11.41
11	[MIF-1 sLeu^2]	173	9.67
12	[MIF-1 sLys^2]	111.1	4.18
13	[MIF-1 sNLe^2]	260	7.29
14	[SA-MIF-1]	228	19.09
15	[SA-MIF-1 Cav$_2$]	145	7.6
16	[SA-Tyr-MIF-1]	146	20.24
17	[SA-Tyr-MIF-1 Cav^3]	135	9.63
18	[SA-Tyr-MIF-1 sLys^3]	170	13.4
19	[Tyr (Br)$_2$-MIF-1]	264	17.93
20	[Tyr (Cl)$_2$-MIF-1]	188	19.55
21	[Tyr (Me)$_2$-MIF-1]	208	19.58
22	[Tyr-MIF-1]	246	18.71
23	[MIF-1 sArg2]	307	20.99
24	[MIF-1 NsArg2]	291	20.18
25	[Tyr-MIF-1 sArg3]	311	21.37
26	[Tyr-MIF-1 NsArg3]	430	28.35

In red are the predicted values for the pain threshold

The analogues were prepared by the classical approach for the synthesis in solution. Procedures were carried out either in homogeneous (organic) or mixed (organic/aqueous) phase. The intermediates were isolated and purified. After completion of the reaction, the protective groups were deblocked and the final peptides were obtained in good yields after column purification (Silica gel 60, CH_3CN:H_2O, 4:1).

For the preparation of the novel peptide analogues (Fig. 3), we used synthetic routes based on the application of the methods of peptide synthesis in solution—mixed anhydrides and activated esters, with the use of the Z-group to protect an α-amino group and benzyl and methyl esters for the carboxyl group protection. A strategy of the

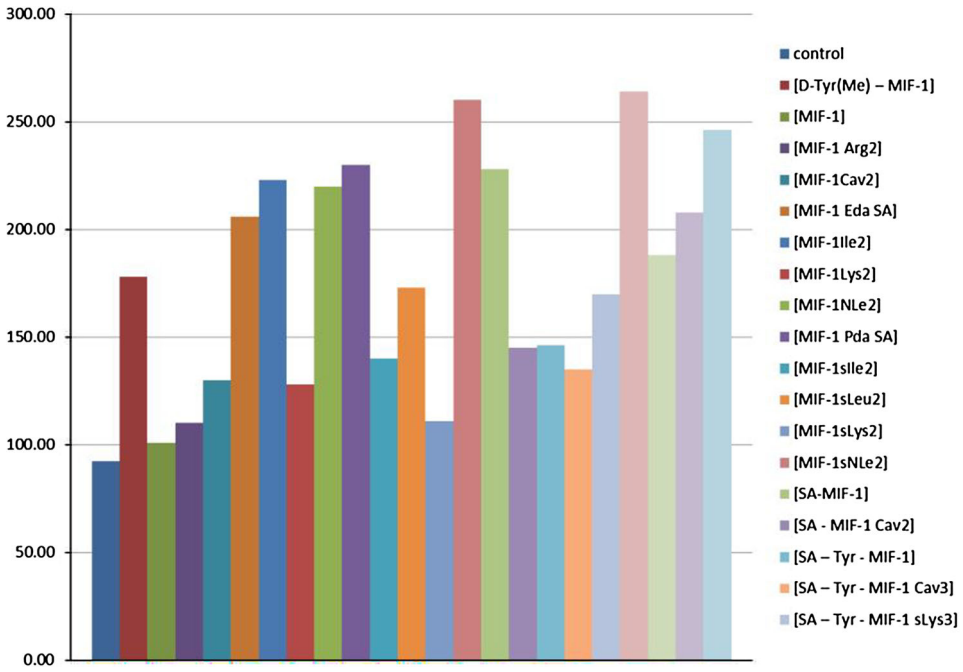

Fig. 2 Effects of MIF-1 and Tyr-MIF-1 analogues in paw-pressure test

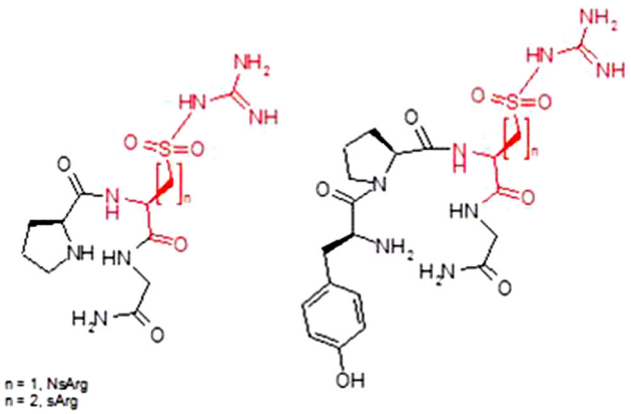

Fig. 3 Structures of MIF-1 and Tyr-MIF-1 analogues with sulgoanalogues of arginine (NsArg—norsulfoarginene and sArg—sulfoarginine) at the position *2* and *3*, respectively

minimum protection of the side chains was used, as in our case the phenolic group of tyrosine and sulfoguanidino group sArg (NsArg) remained unprotected. Synthetic schemes selected to obtain the desired peptides are presented in Figs. 4 and 5.

The scheme for the synthesis of MIF-1 analogues illustrated in Fig. 4 has been successfully applied. The first step of the scheme involved the condensation of Z-Pro-OH and HBr.sArg-OBzl (HBr.NsArg-OBzl) by the method of symmetrical anhydrides using EDAC.HCl. The fully protected MIF-1 analogue was obtained after alkaline hydrolysis of the benzyl ester of the dipeptide and subsequent condensation by the method of symmetrical anhydrides using pivaloyl chloride. The deprotection of the amino group of the tripeptide was achieved by acidic hydrolysis with HBr/acetic acid. The final tripeptide was obtained in good yields after purification by column chromatography.

For the preparation of the tetrapeptide the scheme shown in Fig. 5 was applied. Initially the dipeptides, which connect to each other, were obtained. Z-Tyr-Pro-OH was prepared by the method of activated esters, the condensation of Z-Tyr-OSu and HCl *Pro-OMe in DMF at pH 9, which was maintained by addition of DIPEA. After alkaline hydrolysis it was coupled with sArg-Gly-NH_2 (NsArg-Gly-NH_2). Accordingly it is obtained by the method of mixed anhydrides using Piv-Cl. Its amino protecting group was removed with HBr/CH_3COOH. Tetrapeptide was prepared by the method of mixed anhydrides in the presence of Piv-Cl, and the Z-group is cleaved with HBr/CH_3COOH. The final tetrapeptide is obtained in good yields after purification by column chromatography.

The structures of 26 peptides: MIF-1, Tyr-MIF-1 and analogues thereof, have been generated and optimized using Avogadro and were applied in the docking procedure using GOLD 5.1. For the purpose of docking the crystal structure of μ-opioid receptor was obtained from RCSB (PDB id: 4dkl). This structure was prepared for the procedure, by removing the co-crystallized ligand, by protonating at physiological pH (7.4) and optimizing. From the literature, it is known binding sites of the receptor. These are the residues within a radius of about 10 Å of the aspartic acid residue located in the transmembrane helix 3 or in this case Asp147. ChemScore function was used,

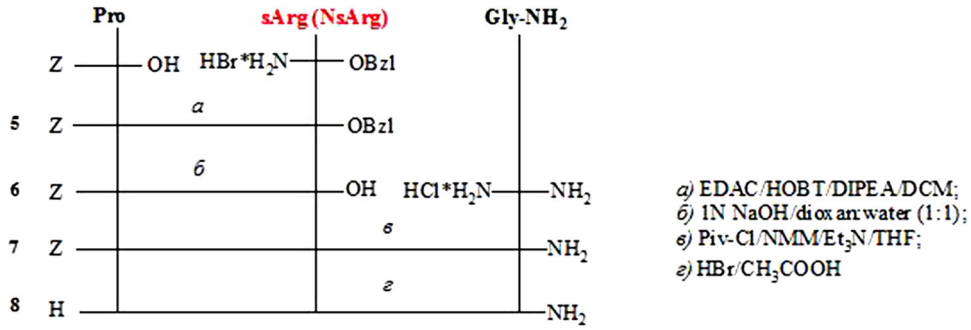

Fig. 4 Synthesis of MIF-1 analogues

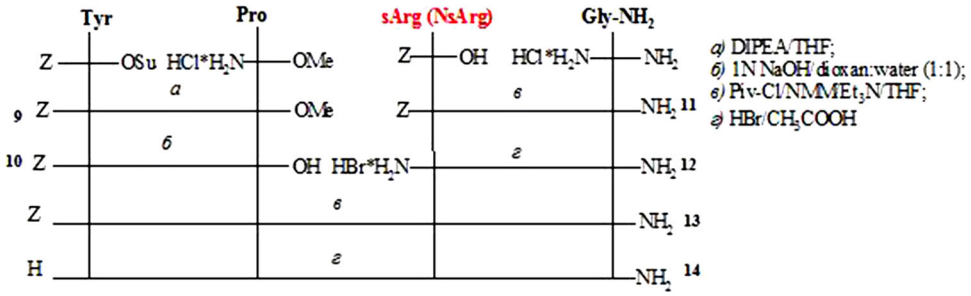

Fig. 5 Synthesis of Tyr-MIF-1 analogues

which makes it possible to verify the ability the corresponding ligand to bind to the receptor.

The values of the scoring function shown in Table 1 indicate that there is a correlation between the ability of the ligand to bind to the receptor and its analgesic effect. In order to establish the relationship between the evaluation function and the data obtained for biological activity, a correlation between these two sets of data is searching for. The correlation analysis is performed using GraphPad Prism®, and the dependence is of the type represented in Fig. 6. A value of the Pearson's coefficient is 0.5218. The function describing the linear relationship between the pain tolerance and the evaluation function can be written in the form:

$$y = 0.0586x + 3.1234. \quad (1)$$

These values indicate that the correlation between the two sets of data is significant and we can talk about the linear relationship between the values of the scoring function and the values of the in vivo test. In other words, it can be said that an increase of the value of the scoring function increases the affinity of the ligand to the receptor, respectively, and hence increases its biological effects.

In order to achieve an effective interaction between the ligand and the receptor, the residue Asp147 is necessary to participate in the formation of a salt bridge with the

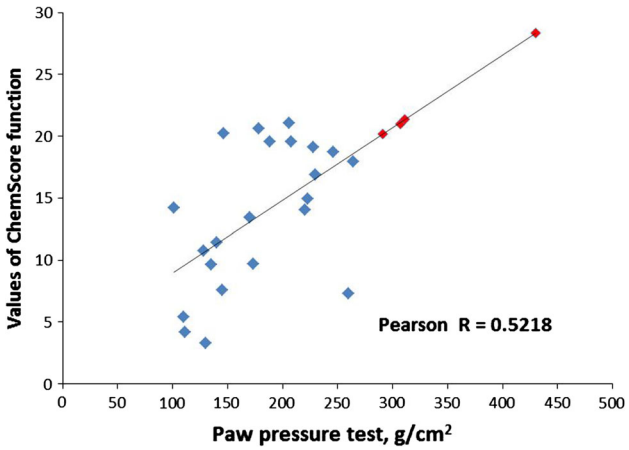

Fig. 6 The correlation between the values of the ChemScore function and biological activity. The *red dots* represent the predicted values of the pain threshold of the newly synthesized analogues (Color figure online)

protonated amino group of the ligand, as in the case of morphine and other endogenous opioid peptides (Kane *et al.,* 2006). In the tested series of analogues of the MIF-1 and Tyr-MIF-1, and MIF-1 and Tyr-MIF-1 themselves, such an interaction was not observed, except with the most active MIF-1 analogue modified in the second position with norsulfoleucine. Another key amino acid residue of the receptor sequence is Tyr148, which forms hydrogen bonds with the active ligands. Tyr-MIF-1 forms such a

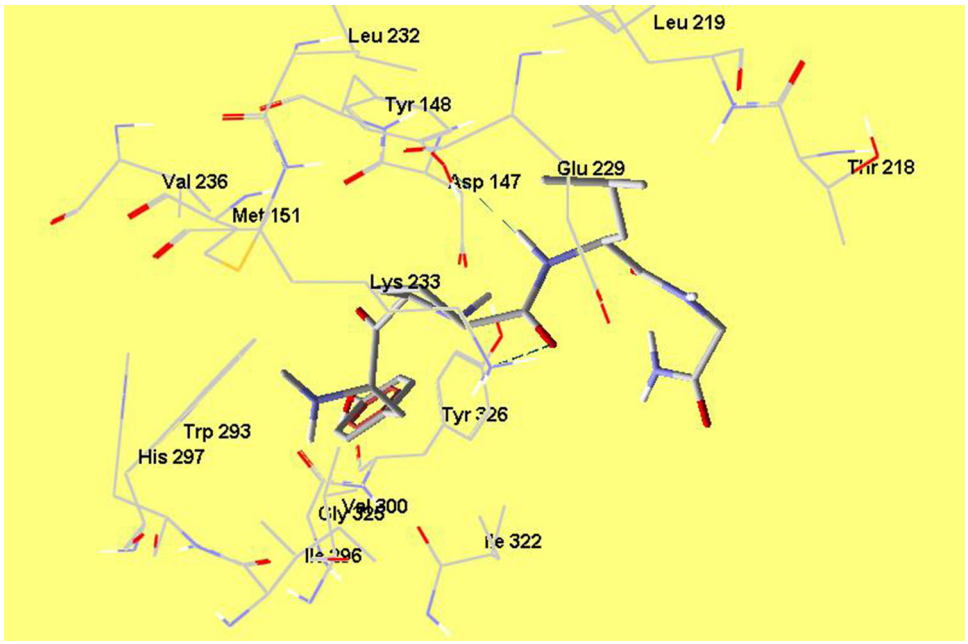

Fig. 7 Interactions of Tyr-MIF-1 with a binding centre of μ-opioid receptor

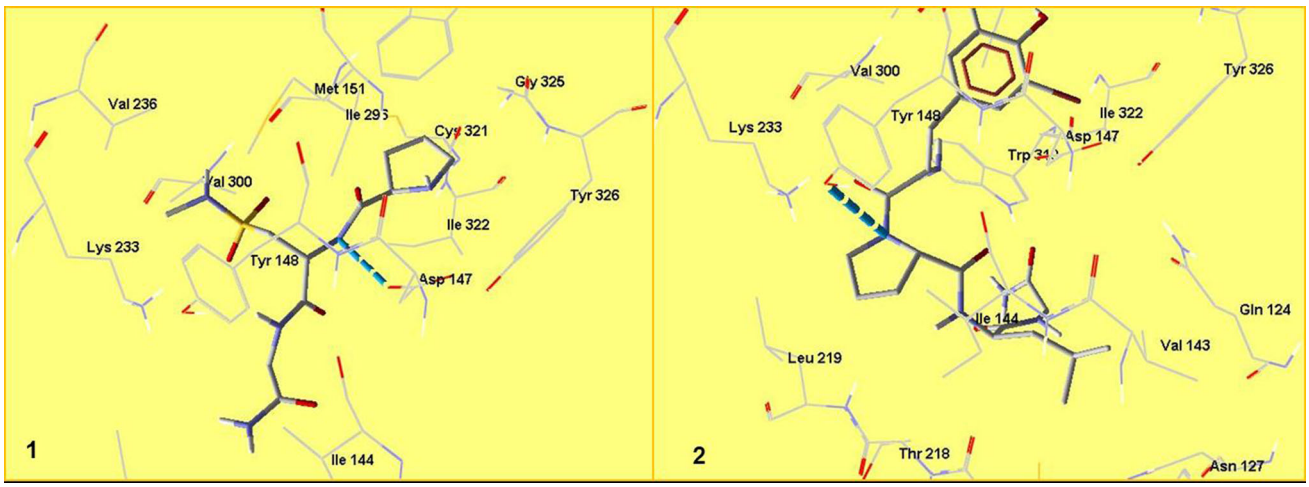

Fig. 8 Binding pocket of μ-opioid receptor with MIF-1 sNLeu^2 (**1**) and Tyr(Br)2-MIF-1 (**2**)

hydrogen bond by its phenolic oxygen atom with a nitrogen atom of the peptide bond between proline and leucine. Leu233 located in the receptor binding centre forms a hydrogen bond with the ζ-NH_2-group and electron withdrawing group of the ligand molecule. In the case of the Tyr-MIF-1, this group is C=O of the peptide bond between proline and leucine (Fig. 7).

The most highly active analogues in the series interact strongly with the receptor, forming hydrogen bonds with the key amino acid residues of the receptor sequence (Fig. 8). MIF-1 sLeu^2 forms a hydrogen bond with Asp147 residue, and Tyr$(Br)_2$-MIF-1—with the Tyr148 residue of the amino acid sequence of the receptor. Tyr$(Cl)_2$-MIF-1 binds Tyr148 and as a result has a strong analgesic effect. Methylation of the free amino group of tyrosine Tyr-MIF-1does not lead to a strong reduction of activity of the analogue, since the values in the in vivo tests did not change significantly. The result of the docking showed that this analogue also successfully connects via a hydrogen bond with the receptor Tyr148. However, replacement of D-Tyr leads to a serious reduction of the effect which can be observed in the results of the docking. D-Tyr (Me)-MIF-1 does not form a hydrogen bond with the receptor, and the only interaction between the ligand and receptor are steric. Coupling with a residue of a cinnamic acid leads to an analogue with a larger molecule. This hampers the binding

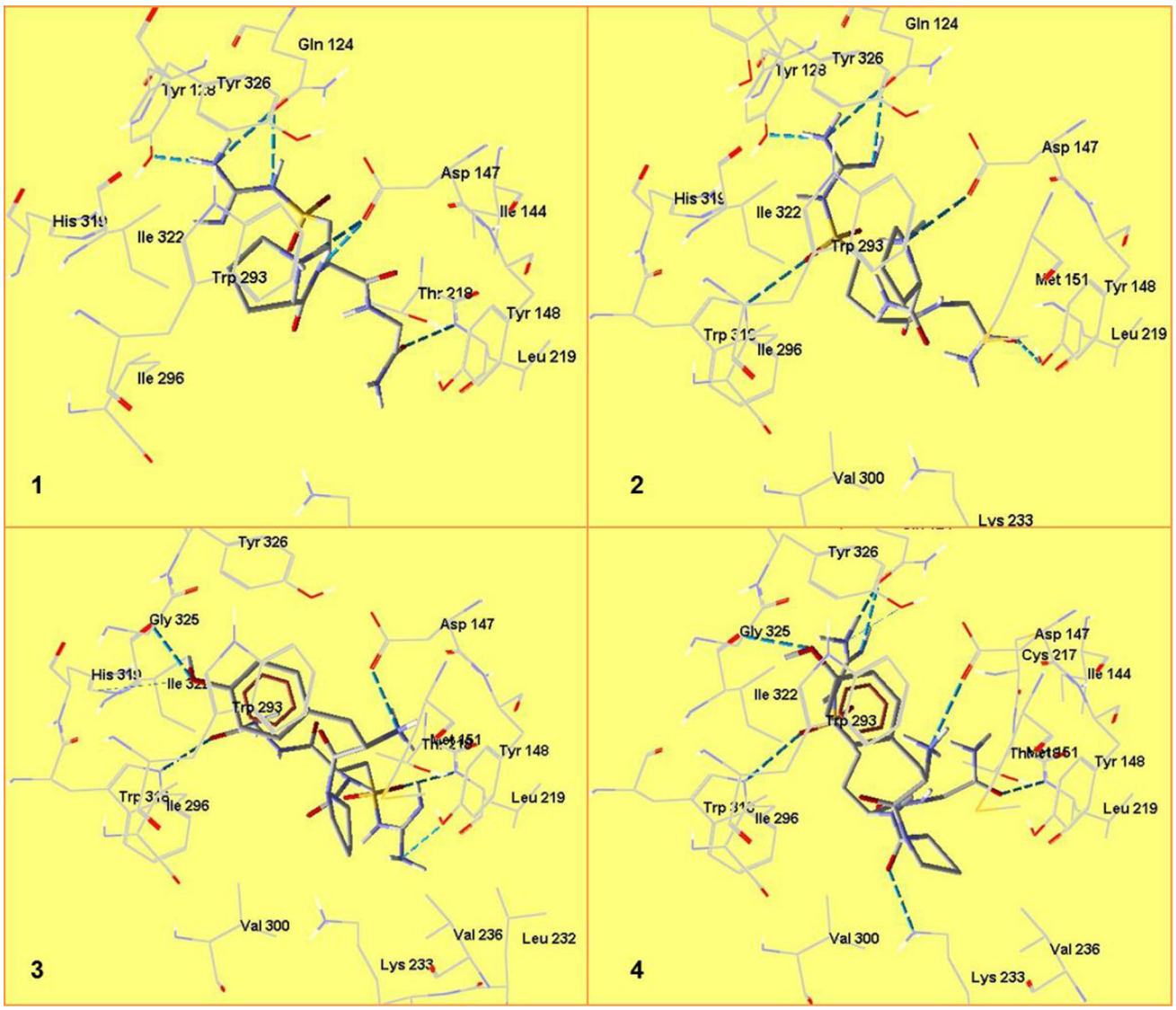

Fig. 9 Binding pocket of the μ-opioid receptor with (**1**) MIF-1 NsArg[2], (**2**) MIF-1 sArg[2], (**3**) Tyr-MIF-1 NsArg[3], (**4**) Tyr-MIF-1 sArg[3]

interaction in the centre of the receptor as a result of steric hindrance. The lack of a free amino group further prevents the recognition of the molecule by the receptor. The reduced biological activity of these analogues is illustrated by the docking results received. Hydrogen bonds with the important amino acid residues in the receptor sequence did not appear. The modified analogues of the MIF-1 with a diamine and cinnamic acid at the C-terminus exert a strong antinociceptive effect. The molecules are again larger than the endogenous MIF-1 and Tyr-MIF-1, but the amino group at the N-terminus of the peptide is intact and receptor can recognize and bind to the corresponding analogue.

The results obtained from the docking for the series of MIF-1 and Tyr-MIF-1 analogues show that it is possible to apply them to the elucidation of the biological effect of various compounds. The dependencies found would be used in subsequent design of new analogues of endogenous peptides MIF-1 and Tyr-MIF-1. If there is a linear relationship between the data from the docking and in vivo experiments when the scoring function is known it can be easily assumed what will be the biological activity of the new analogue. This would reduce the cost of synthesis and biological tests, and only the analogues showed only appropriate value of the scoring function will be synthesized and tested.

In this case, the dependence found was tested in newly synthesized analogues of MIF-1 and Tyr-MIF-1 with modifications in the second and third position, respectively, with sArg and NsArg. The data from the docking are shown in Table 1, as in the column of biological activity is

shown above estimates, calculated by means of the function found. From the data in the table, it is seen that the newly synthesized analogues have very high values of the evaluation function compared with those of the MIF-1, Tyr-MIF-1 and analogues thereof. That alone shows that these peptides would have a high affinity for μ-opioid receptors. Using Eq. (1) with known values of the scoring function is found the corresponding values for the pain threshold and these values are shown in Table 1. Graphically these results are presented in Fig. 6. With the red dots are the predicted values for four new MIF-1 and Tyr-MIF-1 analogues.

Analysing the docking results, it is clear that the newly synthesized analogues bind strongly to the μ-opioid receptor by forming of many hydrogen bonds (Fig. 9). They all interact with Asp147 which is crucial for the analgesic effect. Moreover, they form multiple hydrogen bonds with residues of the receptor binding site. The number of hydrogen bonds between the receptor and ligands are as follows: MIF-1 $NsArg^2$—6, MIF-1 $sArg^2$—7, Tyr-MIF-1 $NsArg^3$—6 and Tyr-MIF-1 $sArg^3$—8. The results obtained indicate that replacement of leucine in the second position within the structure of MIF-1 and in the third position in the Tyr-MIF-1 with sulfo- analogues of arginine leads to the highly active peptides. There is a tendency to increase the biological activity as a result of substitution of leucine residue in the structures of MIF-1 and Tyr-MIF-1 with sulfoanalogue of a natural amino acid. The calculated values for pain threshold will be verified by in vivo tests in order to verify the found dependency. Probably tests would confirm the theoretical values found, since data from the analyses of neuropeptides modified with sulfoanalogues (NsArg and sArg) of arginine show that these analogues exhibit potent analgesic effect (Bocheva *et al.,* 2006). Sulfoanalogues of natural amino acids (Arg, Leu, Lys, NLe) mimetics them good, and there is a sulfo group in the molecule, a new centre, which provides a further possibility of coupling the ligand to the receptor. This further enhances the biological effect.

By analysing the received data from the docking, we have found that this method allows analysis of the data of biological activity at the molecular level. Docking and in particular the analysis of the results of docking allow to look into the mechanism of ligand–receptor interaction, and could be used to elucidate the structure–activity. In this case, we could draw the following conclusions: (1) Modifications of the structure of the MIF-1 and Tyr-MIF-1 analogues lead to an analogue with higher biological activity; (2) Sulfoanalogues of natural amino acids may be successfully applied as modifiers in a variety of peptides, as analogues of the resulting peptides exhibit potent biological effect; (3) Halogenated analogues of Tyr-MIF-1 can successfully mimic the action of native peptides as biological effect is comparable; (4) Methylation of the free amino group of the peptide does not result in a significant change in biological effect; (5) The substitution of an amino acid with its D-isomer causes the reductions a biological effect of the peptide; (6) Coupling with polyamines and cinnamic acid at the C-terminus of the MIF-1 increased the analgesic effect, possibly due to the synergistic effect of the cinnamic acid and MIF-1; and (7) Coupling with cinnamic acid at the N-terminus of the peptide resulted in analogues with decreased biological activity, presumably due to the lack of free amino group necessary for the recognition of the ligand by the receptor.

The fact that there is a correlation between the data obtained from the docking and from an in vivo test allows their use for the prediction of the biological activity of the newly synthesized compounds.

Conclusion

In summary, four new analogues of MIF-1 and Tyr-MIF-1 containing sArg and NsArg were synthesized using well-known methods of peptide synthesis in solution. We have demonstrated that MIF-1 and Tyr-MIF-1 analogues with different modifications exerted strong naloxone-reversible antinociceptive effects in in vivo test.

The molecular docking procedure was applied to determine the interactions between studied ligands and the receptor. From the correlation found between the docking data and the in vivo test, we could predict the biological effect of newly synthesized analogues, which is much higher than other compounds in the tested series. Applying the docking procedure, we could make prediction about the biological effects of newly synthesized compounds, and we could propose structures with higher biological activity.

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