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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Kyotorphin analogues containing unnatural amino acids: synthesis, analgesic activity and computer modeling of their interactions with μ-receptor

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Abstract Kyotorphin (KTP; Tyr-Arg) an endogenous neuropeptide is potently analgesic when delivered directly to CNS. An effort to enhance the potency, enzymatic stability and improving bioavailability of KTP is the modification with unnatural amino acids. The aims of presented study were: (1) To synthesize new analogues of kyotorphin containing unnatural amino acids: norcanavaine (NCav) and norcanaline (NCan), structural analogues of arginine and ornithine, respectively; (2) To understand the influence of the arginine mimetics on the pharmacological properties of KTP analogues, through examination their effects on the paw pressure nociceptive threshold; (3) To find relationship between the structure and obtained biological effects of the all synthesized kyotorhin analogues, by molecular docking with μ-opioid receptor. As a result of our work four new kyotorphin analogues containing NCan and NCav were obtained. A correlation between the data from the in vivo test and docking results was found. This allows a better elucidation of the ligand-receptor interactions, the prediction of biological activity of the newly synthesized compounds, and to generate compounds with increased biological effects.

Keywords Arginine mimetics · Kyotorphin · Nociception · μ-Opioid receptor · Docking · GOLD

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Abbreviations

KTP	Kyotorphin
CNS	Central nervous system
MOR	μ-Opioid receptors
PP	Paw-pressure nociceptive test
Cav	Canavanine
NCav	Norcanavanine
Can	Canaline
NCan	Norcanaline
NsArg	Norsulfoarginine
Piv-Cl	Pivaloyl chloride
DIPEA	*N,N*-diisopropylethylamine
Boc	Di-tert-butyl dicarbonate
Z	Carboxybenzyl

Introduction

Kyotorphin (KTP; L-Tyr-L-Arg) was first isolated from bovine brain in 1979 (Takagi *et al.*, 1979) and has subsequently been found in the brain of several mammals and in human cerebrospinal fluid (Kolaeva *et al.*, 2000).

The most studied characteristics of KTP are its structure, biosynthesis and hydrolysis, the mechanisms of its release from nerve terminals, its interaction with the postsynaptic membrane and the binding to specific receptors (Kawabata *et al.*, 1996, 1993). Kyotorhin is an endogenous dipeptide and may be formed by the biosynthesis from Tyr and Arg (Ueda *et al.*, 1982). It can be consider as a neurotransmitter, satisfied all five criteria (Zakutskii *et al.*, 2008): (1) it is synthesized in synaptic endings; (2) it is stored in synaptic vesicles (Ueda *et al.*, 1982); (3) it is released at the depolarization of presynaptic membranes (Ueda *et al.*, 1986a), (4) it is enzymatically inactivated by kyotorphin hydrolase (Ueda *et al.*, 1985), and (5) KTP interacts with

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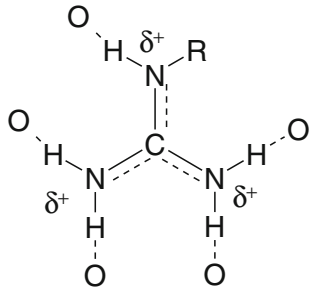

Fig. 1 Schematic presentation of guanidinium group hydrogen bond formation with five different oxygen atoms

specific kyotorphin receptors on the postsynaptic membrane. This leads to the activation of phospholipase C, and an increase in the concentration of inositol-3-phosphate (Ueda *et al.*, 1985). These properties of KTP are typical for the neurotransmitters, and it exerts nonopioid actions independent of enkephalin release (Ueda and Inoue, 2000). This fact point out the possibility of an indirect action of KTP, which would not bind to the opioid receptors (μ, δ, κ), but it is involved in Met-enkephalin release (Takagi *et al.*, 1979). All this led to presumption that the dipeptide bind to a specific receptor (kyotorphin receptor, KTPr) (Ueda *et al.*, 1986b), triggering a cascade of events that could cause strong analgesia in the brain (Ueda *et al.*, 1987, 2000). Despite several studies (Takagi *et al.*, 1979; Börjesson and Hünenberger, 2001; Shiomi *et al.*, 1981) confirmed the existence of kyotorphin receptor, it has not yet been identified. There is still the question of whether the kyotorphin receptor is specific (Börjesson and Hünenberger, 2001), or it is the result of a mixed oligomerization of μ- and δ-opioid receptors (George *et al.*, 2000).

Even it is known that kyotorphin's binding pocket has to be different from the opioid receptors (Ueda *et al.*, 2000; Takagi *et al.*, 1979; Hansen *et al.*, 1992), there are obvious similarities between KTP and most of the endogenous opioid peptides and even with morphine (Machuqueiro and Baptista, 2007). The L-Tyr residue at the first position of the peptide is present in most of the opioid peptides, and is believed to be crucial for receptor recognition due to both π-stacking and hydrogen-bonding with the alcohol of the phenol group. A protonable N-terminal group is also present in KTP and when protonated, can form a salt bridge with an anionic group in the receptor, which is typical for the morphine/μ-receptor interaction. Conformational studies of kyotorphin molecule (Machuqueiro and Baptista, 2007) suggest that, despite the fact that kyotorphin does not bind to the opioid receptors, there is no indication for the kyotorphin receptor pocket to be very different from those of the opioid receptors.

The L-Arg residue at the second position of kyotorphin has guanidinium group, which is positively charged at neutral pH and is involved in many important physiological and pathophysiological processes. The guanidinium group of arginine has a $pK_a = 12$, has planar geometry and can be a donor in the formation of up to five hydrogen bonds (Fig. 1).

The arginine has been found to play an important role in binding of negatively charged compounds such as: substrates; cofactors; and different effectors to the protein active sites (Riordan *et al.*, 1977). It is often involved in formation of salt bridges, mainly with aspartate or glutamate side chains. The arginine also plays numerous roles in the cell metabolism (Fig. 2). Endogenously applied, L-Arg acts as an effective precursor of kyotorphin and has an antinociceptive effect in rats and mice (Kawabata *et al.*, 1993, 1996). Additionally, clinical studies demonstrated naloxone-reversible analgesic effects of L-Arg in chronic pain patients (Harima *et al.*, 1991).

Despite its interesting properties, KTP is only active when injected directly into the brain. When systemically administered, it only shows brief activity and at a high dose of 200 mg kg^{-1} (Chen *et al.*, 1998). This limited capacity to cross the blood–brain barrier, mainly related to both insufficient lipophilicity and susceptibility to various lytic enzymes, confines KTP pharmacological applications. A logical strategy to target KTP to the brain is the chemical modification of KTP into analogues that are able to maintain analgesic properties and are able to cross the blood–brain barrier.

We started to search for a KTP derivative with minimal differences relative to KTP, focusing our attention on the replacement of arginine with its analogues with reduced basicity. Many efforts were made in the design and preparation of arginine analogues with reduced basicity, resulting in increased stability towards enzymatic degradation (Peterlin-Mašič and Kikelj, 2001). In view of this, it was interesting to design and synthesize unnatural amino acids containing a guanidinium functionality (sulfo- and oxyguanidinium), as structural analogue of arginine (Pajpanova *et al.*, 1997; Stanchev *et al.*, 2000; Dzimbova *et al.*, 2003, 2007). The synthesis and evaluation of kyotorhins containing these arginine analogues was done. Modification in the arginine residue of kyotorphin with canavanine and sulfoarginine has also been shown to have a significant influence on the analgesic activity (Bocheva *et al.*, 1996; Dzimbova *et al.*, 2006).

In the present paper we describe a several of kyotorhins that we designed with the goal to develop analogues with increased degradation stability and potency. The design was mainly based on structural modification of Arg residue. Arg^2 was replaced with unnatural amino acids (NCav and NCan), structural analogues of canavanine and canaline, respectively. The reverse-kyotorphins were also prepared. In order to understand the influence of the arginine mimetics on the pharmacological properties of KTP

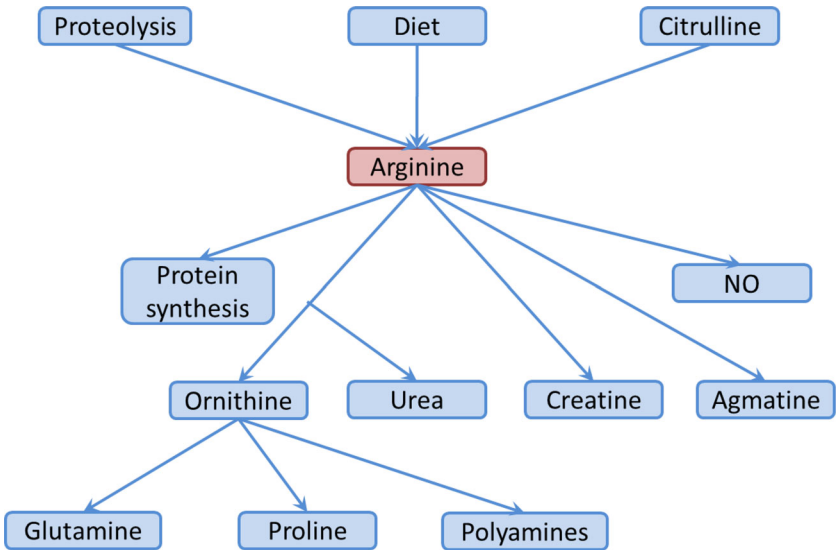

Fig. 2 Arginine metabolism

analogues, we studied their effects on the PP (paw-pressure) nociceptive threshold. Next, assumed to find relationship between the structure and obtained biological effects of the all synthesized kyotorhin analogues, molecular docking with μ-opioid receptor was applied. The elucidation of ligand-receptor interaction would be helpful for the discovery of new active analogs of KTP.

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 of 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 MHz and 75 MHz, respectively) spectrometers. Mass spectra were recorded on a Fissons-Triple Quadrupol-ES mass spectrometer. The IR-spectra were measured using a Bomem-Michelson 100 FT-IR-spectrometer (4,000–400, 2 cm^{-1} resolution, 150 scans). Solid-state IR spectra were recorded, using the KBr disk technique.

Amino acids and analogues

Amino acids D-Kyo, Kyo, L-Can, L-Cav, L-Cit, L-NAME, and L-Orn were obtained from Sigma. The Cys(O_2NH_2) was synthesized following original synthetic scheme presented by Aleksiev and Stoev (1971). The synthesis of unnatural amino acids: L-NCan; NsArg; NsArg-NH_2 and NsArg-OBzl (Dzimbova *et al.*, 2003, 2011) were previously reported.

Peptide synthesis

The synthesis of the kyotorphin analogues: Tyr-Cav (Bocheva *et al.*, 1996), Tyr(Cl_2)-Cav (Pajpanova *et al.*, 1992) and NsArg-Tyr, Tyr-NsArg, Tyr-NsArg-NH_2, Tyr-NsArg-OBzl (Dzimbova *et al.*, 2006) were previously reported.

Synthesis of Z-NCan-Tyr-OMe (methyl 2-(3-(aminooxy)-2-(((benzyloxy)carbonyl)amino)propanamido)-3-(4-hydroxyphenyl)propanoate) ***(23)*** *and Z-NCav-Tyr-OMe (methyl 2-(2-(((benzyloxy)carbonyl)amino)-3-(guanidinooxy)propanamido)-3-(4-hydroxyphenyl)propanoate)* ***(24)***

A solution of Z-NCan (Z-NCav) (0.5 mM) and NMM (0.06 ml, 0.5 mM) in 5 ml DMF was cooled to −10 °C and Piv-Cl (0.061 ml, 0.5 mM) was added dropwise. After 10 min a solution of HCl.Tyr-OMe (0.11 g, 0.5 mM) and Et_3N (0.07 ml, 0.5 mM) in 5 ml DMF was added. The reaction mixture was stirred 4 h at room temperature. Before it was quenched by the addition of water (10 ml) and extracted with $CHCl_3$ (3 × 10 ml). The combined organic phases were washed consequently with 5 % $NaHCO_3$ (3 × 10 ml), 5 % $NaHSO_4$ (3 × 10 ml), and

brine, dried over anhydrous Na_2SO_4, filtered and $CHCl_3$ was evaporated. The work-up procedure describe above yielded a Z-NCan-Tyr-OMe (0.11 g, 51 %) and Z-NCav-Tyr-OMe (0.14 g, 55 %) as an oils, respectively. **23**: MS-ES, *m/z*: 432 [M^+] (431.44); Anal. Calcd. for $C_{21}H_{25}N_3O_7$: C, 58.46; H, 5.84; N, 9.74; O, 25.96. Found: C, 58.33; H, 5.87; N, 9.79; O, 26.01. ^{1}H-NMR ($CDCl_3$) δ/ppm: 8.03 (s, 2H, NH), 7.47 (m, 2H, Ar), 7.38 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.09 (s, 2H, $PhCH_2$), 4.62 (m, 1H, CH), 4.55 (m, 1H, CH), 4.16 (d, 2H, CH_2), 3.68 (s, 3H, CH_3), 3.23 (d, 2H, CH_2), 2.0 (s, 2H, NH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 171.5, 170.4, 155.9, 155.7, 136.1, 130.2, 129.2, 128.9, 127.6, 127.1, 115.8, 76, 66.8, 56.7, 55.9, 51.9, 36.8; IR (KBr) cm^{-1}: 3371, 3062, 2950, 2424, 1750, 1582, 1454, 1480, 1368, 1255, 1122, 933, 762; **24**: MS-ES, *m/z*: 474 [M^+] (473.48); Anal. Calcd. for $C_{22}H_{27}N_5O_7$: C, 55.81; H, 5.75; N, 14.79; O, 23.65. Found: C, 55.72; H, 5.7; N, 14.72; O, 23.86.^{1}H-NMR ($CDCl_3$) δ/ppm: 8.58 (s, 3H, Gu), 8.03 (s, 2H, NH), 7.47 (m, 2H, Ar), 7.38 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.09 (s, 2H, $PhCH_2$), 4.62 (m, 1H, CH), 4.55 (m, 1H, CH), 4.16 (d, 2H, CH_2), 3.68 (s, 3H, CH_3), 3.23 (d, 2H, CH_2), 2.0 (s, 2H, NH); ^{13}C-NMR ($CDCl_3$) δ/ppm:171.5, 170.4, 158.5, 155.9, 155.7, 136.1, 130.2, 129.2, 128.9, 127.6, 127.1, 115.8, 73.6, 66.8, 56.7, 55.9, 51.9, 36.8; IR (KBr) cm^{-1}: 3000–2356, 1692, 1535, 1457, 1333, 1255, 1160, 1073, 983, 899, 704.

Synthesis of Boc-Tyr-NCan-OBzl (benzyl 3-(aminooxy)-2-(2-((tert-butoxycarbonyl)amino)-3-(4-hydroxyphenyl) propanamido)propanoate) ***(25)*** *and Boc-Tyr-NCav-OBzl (benzyl 2-(2-((tert-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)propanamido)-3-(guanidinooxy)propanoate)* ***(26)***

A solution of 2HBr.NCan-OBzl (2HBr.NCav-OBzl) (1.1 mM) and DIPEA (0.4 ml, 2.2 mM) in 5 ml DMF was added to a solution of Boc-Tyr-OSu (0.42 g, 1.1 mM) in 5 ml DMF. Reaction was carried out at room temperature for 24 h. After complete the reaction 10 ml of water was added followed by extraction with $CHCl_3$ (3 × 10 ml). Combined organic layers were washed consequently with 5 % $NaHCO_3$ (3 × 10 ml), 5 % $NaHSO_4$ (3 × 10 ml), and brine, dried over anhydrous Na_2SO_4 and $CHCl_3$ was evaporated. The work-up procedure describe above yielded a 0.35 g (67 %) of Boc-Tyr-NCan-OBzl and 0.37 g (65 %) of Boc-Tyr-NCav-OBzl, respectively. **25**: MS-ES, *m/z*: 474 [M^+] (473.52); Anal. Calcd. for $C_{24}H_{31}N_3O_7$: C, 60.88; H, 6.60; N, 8.87; O, 23.65. Found: C, 60.82; H, 6.67; N, 8.85; O, 23.66.^{1}H-NMR ($CDCl_3$) δ/ppm: 8.03 (s, 2H, NH), 7.47 (m, 2H, Ar), 7.38 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.34 (s, 2H, $PhCH_2$), 4.92 (m, 1H, CH), 3.44 (s, 2H, $PhCH_2$), 4.26 (d, 2H, CH_2), 3.98 (m, 1H, CH), 3.44 (d, 2H, CH_2), 2.0 (s, 2H, NH_2), 1.38 (s, 9H, CH_3); ^{13}C-NMR ($CDCl_3$) δ/ppm:171.7, 169.1, 155.9, 155.7, 136.1, 130.2, 129.2, 128.9, 127.6, 127.1, 115.8, 79.5, 75.5, 66.4, 63.3, 58.8, 37.3, 28.4; IR (KBr) cm^{-1}: 3300–2920, 1698, 1535, 1451, 1410, 1332, 1259, 1134, 1090, 975, 910, 883; **26**: MS-ES, *m/z*: 516 [M^+] (515.56); Anal. Calcd. for $C_{25}H_{33}N_5O_7$: C, 58.24; H, 6.45; N, 13.58; O, 21.72. Found: C, 58.29; H, 6.40; N, 13.61; O, 21.70.^{1}H-NMR ($CDCl_3$) δ/ppm: 8.58 (s, 3H, Gu), 8.03 (s, 2H, NH), 7.47 (m, 2H, Ar), 7.38 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.34 (s, 2H, $PhCH_2$), 4.92 (m, 1H, CH), 4.26 (d, 2H, CH_2), 3.98 (m, 1H, CH), 3.44 (d, 2H, CH_2), 2.0 (s, 2H, NH), 1.38 (s, 9H, CH_3); ^{13}C-NMR ($CDCl_3$) δ/ppm:171.7, 169.1, 158.5, 155.9, 155.7, 136.1, 130.2, 129.2, 128.9, 127.6, 127.1, 115.8, 79.5, 73.1, 66.4, 63.3, 58.8, 37.3, 28.4; IR (KBr) cm^{-1}: 3350–2928, 1640, 1592, 1404, 1253, 1155, 1062, 974, 879, 803.

Synthesis of Z-NCan-Tyr-OH (2-(3-(aminooxy)-2-(((benzyloxy)carbonyl)amino)propanamido)-3-(4-hydroxyphenyl)propanoic acid) ***(27)****, Z-NCav-Tyr-OH (2-(2-(((benzyloxy)carbonyl)amino)-3-(guanidinooxy)propanamido)-3-(4-hydroxyphenyl)-propanoic acid)* ***(28)****, Boc-Tyr-NCan-OH (3-(aminooxy)-2-(2-((tert-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)-propanamido)propanoic acid)* ***(29)****, and Boc-Tyr-NCav-OH (2-(2-((tert-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)propanamido)-3-(guanidinooxy)propanoic acid)* ***(30)***

The peptide **23** (**24**, **25**, **26**) (0.2 mM) was dissolved in a mixture of dioxane: water (1: 1, 10 ml), and drop of thymolphthalein in MeOH was added. Then a solution of 1 N NaOH was added dropwise until complete saponification (blue color of the solution was constant). The dioxane was evaporated, and the residue was extracted with EtOAc (3 × 10 ml), acidified to pH 3 with dry $NaHSO_4$ and extracted again with EtOAc (3 × 10 ml). Combined organic layers were washed with water, dried over anhydrous Na_2SO_4, filtered and the EtOAc was evaporated. The work-up procedure describe above yielded a Z-NCan-Tyr-OH (0.059 g, 72 %), Z-NCav-Tyr-OH (0.087 g, 68 %), Boc-Tyr-NCan-OH (0.02 g, 23 %), and Boc-Tyr-NCav-OH (0.04 g, 44 %), respectively. **27**: MS-ES, *m/z*: 418 [M^+] (417.41); Anal. Calcd. for $C_{20}H_{23}N_3O_7$: C, 57.55; H, 5.55; N, 10.07; O, 26.83. Found: C, 57.50; H, 5.57; N, 10.11; O, 26.82.^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s, 1H, OH), 8.03 (s, 2H, NH), 7.47 (m, 2H, Ar), 7.38 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.09 (s, 2H, $PhCH_2$), 4.72 (m, 1H, CH), 4.62 (m, 1H, CH), 4.16 (d, 2H, CH_2), 3.12 (d, 2H, CH_2), 2.0 (s, 2H, NH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 174.7, 170.4, 155.9, 155.7, 136.1, 130.2, 129.2, 128.9, 127.6, 121.1, 115.8, 76.0, 66.8, 59.2,

55.9, 36.5; IR (KBr) cm^{-1}: 3380–2880, 1746, 1528, 1403, 1253, 1176, 1068, 934, 715; **28**: MS-ES, *m/z*: 460 [M^+] (459.45); Anal. Calcd. for $C_{21}H_{25}N_5O_7$: C, 54.90; H, 5.48; N, 15.24; O, 24.38. Found: C, 54.93; H, 5.42; N, 15.28; O, 24.37.^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s, 1H, OH), 8.58 (s, 3H, Gu), 8.03 (s, 2H, NH), 7.47 (m, 2H, Ar), 7.38 (m, 3H, Ar), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.09 (s, 2H, $PhCH_2$), 4.72 (m, 1H, CH), 4.62 (m, 1H, CH), 4.16 (d, 2H, CH_2), 3.12 (d, 2H, CH_2), 2.0 (s, 2H, NH); ^{13}C-NMR ($CDCl_3$) δ/ppm: 174.7, 170.4, 158.5, 155.9, 155.7, 136.1, 130.2, 129.2, 128.9, 127.6, 121.1, 115.8, 73.6, 69.2, 66.8, 55.9, 36.5; IR (KBr) cm^{-1}: 3667–3010, 2930, 1708, 1405, 1257, 1133, 1073, 900, 805; **29**: MS-ES, *m/z*: 384 [M^+] (383.40); Anal. Calcd. for $C_{17}H_{25}N_3O_7$: C, 53.26; H, 6.57; N, 10.96; O, 29.21. Found: C, 53.29; H, 6.52; N, 10.94; O, 29.25. ^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s, 1H,OH), 8.03 (s, 2H, NH), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 4.92 (m, 1H, CH), 4.14 (d, 2H, CH_2), 4.02 (m, 1H, CH), 3.44 (s, 2 h, $PhCH_2$), 2.0 (s, 2H, NH_2), 1.38 (s, 9H, CH_3); ^{13}C-NMR ($CDCl_3$) δ/ppm: 171.7, 171.0, 155.9, 155.7, 130.2, 129.2, 115.8, 79.5, 75.2, 58.8, 55.5, 37.3, 28.4; IR (KBr) cm^{-1}: 3545–2876, 1644, 1593, 1416, 1179, 1135, 1068, 720; **30**: MS-ES, *m/z*: 426 [M^+] (425.44); Anal. Calcd. for $C_{18}H_{27}N_5O_7$: C, 50.82; H, 6.40; N, 16.46; O, 26.32. Found: C, 50.87; H, 6.43; N, 16.41; O, 26.29. ^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s,1H, OH), 8.58 (s, 3H, Gu), 8.03 (s, 2H, NH), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 4.92 (m, 1H, CH), 4.14 (d, 2H, CH_2), 4.02 (m, 1H, CH), 3.44 (d, 2H, CH_2), 2.0 (s, 2H, NH), 1.38 (s, 9H, CH_3); ^{13}C-NMR ($CDCl_3$) δ/ppm: 171.7, 171.0, 158.5, 155.9, 155.2, 130.1, 129.2, 115.8, 79.5, 72.8, 58.8, 55.5, 37.3, 28.4; IR (KBr) cm^{-1}: 3320–2876, 1706, 1654, 1430, 1260, 1142, 1115, 1036, 900, 715.

Synthesis of HCl.NCan-Tyr (2-(2-amino-3-(aminooxy)propanamido)-3-(4-hydroxyphenyl)propanoic acid) ***(10)*** *and HCl.NCav-Tyr (2-(2-amino-3-(guanidinooxy)propanamido)-3-(4-hydroxyphenyl)propanoic acid)* ***(11)***

The peptide **25** (**26**) (0.1 mM) was dissolved in 1 ml CH_3COOH and HBr/CH_3COOH (0.5 ml) was added. The deprotection continued for 1 h at room temperature. The solvent was evaporated, and the crude product was treated three times with MeOH (3 × 20 ml), which was also evaporated. The obtained HBr-salts of the kyotorphin analogues were transformed to free bases with Et_3N in CH_2Cl_2. The CH_2Cl_2 was evaporated and dipeptides were treated with 3 M HCl/EtOAc to obtain crystal HCl-salts of NCan-Tyr and NCav-Tyr, respectively. Final peptides were obtained after column purification (Silicagel 60, CH_3CN: H_2O, 4:1). **10**: MS-ES, *m/z*: 284 [M^+] (283.28); Anal. Calcd. for $C_{12}H_{17}N_3O_5$: C, 50.88; H, 6.05; N, 14.83; O, 28.24. Found: C, 50.90; H, 6.01; N, 14.87; O, 28.22. ^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s, 1H, OH), 8.03 (s, 1H, NH), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.11 (d, 2H, NH_2), 4.72 (m, 1H, CH), 4.16 (d, 2H, CH_2), 3.65 (m, 1H, CH), 3.12 (d, 2H, CH_2), 2.0 (s, 2H, NH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 174.7, 171.7, 155.7, 130.2, 129.2, 115.8, 78.8, 59.2, 52.0, 36.5; IR (KBr) cm^{-1}: 3550–2960, 1752, 1638, 1403, 1222, 1160, 1079, 898, 738; **11**: MS-ES, *m/z*: 338 [M^+] (337.33); Anal. Calcd. for $C_{14}H_{19}N_5O_5$: C, 48.00; H, 5.89; N, 21.53; O, 24.59. Found: C, 47.95; H, 5.92; N, 21.55; O, 24.58. ^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s, 1H, OH), 8.56 (s, 3H, Gu), 8.03 (s, 1H, NH), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.11 (d, 2H, NH_2), 4.72 (m, 1H, CH), 4.16 (d, 2H, CH_2), 3.65 (m, 1H, CH), 3.12 (d, 2H, CH_2), 2.0 (s, 2H, NH); ^{13}C-NMR ($CDCl_3$) δ/ppm: 174.7, 171.7, 158.5, 155.7, 130.2, 129.2, 115.8, 76.4, 59.2, 52.0, 36.5; IR (KBr) cm^{-1}: 3381–2949, 1740, 1528, 1410, 1230, 1165, 1054, 903, 706.

Synthesis of HCl.Tyr-NCan (2-(2-amino-3-(4-hydroxyphenyl)propanamido)-3-(aminooxy)propanoic acid) ***(18)*** *and HCl.Tyr-NCav (2-(2-amino-3-(4-hydroxyphenyl)-propanamido)-3-(guanidinooxy)propanoic acid)* ***(19)***

The peptide **29** (**30**) (0.4 mM) was dissolved in 2 ml 3 M HCl/EtOAc. During the reaction crystals of the new products were formed. After 1 h reaction was completed and obtained crystal product was filtered and washed with diethyl ether. Final peptides were obtained after column purification (Silicagel 60, CH_3CN: H_2O, 4:1). The work-up procedure describe above yielded a 0.09 g (83 %) of HCl.Tyr-NCan and 0.07 g (81 %) of HCl.Tyr-NCav, respecively. **18**: MS-ES, *m/z*: 284 [M^+] (283.28); Anal. Calcd. for $C_{12}H_{17}N_3O_5$: C, 50.88; H, 6.05; N, 14.83; O, 28.24. Found: C, 50.93; H, 6.00; N, 14.85; O, 28.22. ^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s, 1H,OH), 8.03 (s, 1H, NH), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.11 (d, 2H, NH_2), 4.14 (d, 2H, CH_2), 4.02 (m, 1H, CH), 3.95 (m, 1H, CH), 3.44 (s, 2 h, $PhCH_2$), 2.0 (s, 2H, NH_2); ^{13}C-NMR ($CDCl_3$) δ/ppm: 171.7, 171.0, 155.7, 130.2, 129.2, 115.8, 75.2, 56.0, 55.5, 38.7; IR (KBr) cm^{-1}: 3300–2876, 1679, 1542, 1401, 1266, 1150, 1078, 895, 711; **19**: MS-ES, *m/z*: 338 [M^+] (337.33); Anal. Calcd. for $C_{13}H_{19}N_5O_5$: C, 48.00; H, 5.89; N, 21.53; O, 24.59. Found: C, 47.98; H, 5.85; N, 21.57; O, 24.60. ^{1}H-NMR ($CDCl_3$) δ/ppm: 11.0 (s,1H, OH), 8.56 (s, 3H, Gu), 8.03 (s, 1H, NH), 7.12 (m, 2H, Ar), 6.7 (d, 2H, Ar), 5.35 (s, 1H, Ph-OH), 5.11 (d, 2H, NH_2), 4.14 (d, 2H, CH_2), 4.02 (m, 1H, CH), 3.95 (m, 1H, CH), 3.44 (d, 2H, CH_2), 2.0 (s, 2H, NH); ^{13}C-NMR ($CDCl_3$) δ/ppm: 171.7, 171.0, 158.5, 155.7, 130.2, 129.2, 72.8, 56.0, 55.5, 38.7; IR (KBr) cm^{-1}: 3300–2887, 1647, 1404, 1158, 1075, 884, 714.

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 animal 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 the 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 by 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

We were interested in studying and comparing the effects of the replacement of Arg with various unnatural amino acids on the structural properties and on the biological activity of kyotorphin.

Non-proteinogenic, unnatural α-amino acids have been widely used as modifiers of peptides to enhance biological activity, proteolytic stability, and bioavailability. Our previous efforts were focused on the preparation and the characterization of unnatural amino acids, particularly those containing a basic functionality (amino, oxyamino, sulfoamino, oxyguanidinium and sulfoguanidinium) in the side chain. We synthesized numerous unnatural amino acids structural analogues of arginine such as: sulfoarginines (sArg, NsArg); canavanines (Cav, NCav), and of lysine (sLys, HsLys), which demonstrated certain nociceptive effects (Videnov *et al.*, 1993; Pajpanova *et al.*, 1997; Dzimbova *et al.*, 2011). For instance, in our previous studies (Brakadanska *et al.*, 2001) we demonstrated that L-Cav (at a dose 20 μg/rat, *i.c.v*) exerted strong naloxone-reversible antinociceptive effects Fig. 3).

We examined for possible analgesic activity the synthesized sulfoarginine, its derivatives and canavanine in rats by PP nociceptive test. All amino acids were applied intraperitoneally (i.p.) at a dose of 1 mg kg^{-1}. The investigations started 15 min. after injection of amino acids, and all amino acid analogues showed significant analgesic effects compared to the control (Fig. 3). Tyr-NsArg-NH_2, Tyr-NsArg-OBzl (all at dose 5 mg kg^{-1}) estimated by paw-pressure test (Bocheva *et al.*, 2006). Data are presented as mean ± SEM.

To illustrate the impact of this non-proteinogenic amino acid on bioactivity, we synthesized a number of analogues modified in both positions (Fig. 4) by introducing Cav instead Arg into the sequence. We also studied the antinociceptive activity analgesic effects of Tyr-Cav, Tyr(Cl_2)-Cav on the most frequently used models based on tests with thermal (HP, TF) and mechanical irritation (PP). Changes in the nociceptive effects of the peptides applied (*i.c.v.)* at a dose of 20 μg rat^{-1} in male Wistar rats were examined by (TF) and (HP) tests. The obtained results showed that Kyo, Tyr-Cav and Tyr(Cl_2)-Cav significantly increased the latency in both used nociceptive tests. Tyr-Cav exerted a strong analgesic action by tail-flick test, and the effect is a naloxone-reversible. The effects of Tyr-Cav were more pronounced compared to Kyo (Bocheva *et al.*, 1996).

Further we investigate the possible analgesic activity of synthesized Kyo analogues containing NsArg and derivatives in rats (Bocheva *et al.*, 2006). The antinociceptive effects of all these dipeptides (NsArg-Tyr, Tyr-NsArg-NH_2 and Tyr-NsArg-Bzl) were evaluated after *i.p.* administration in rats. All peptides at a dose of 5 mg kg^{-1} estimated by PP test showed naloxone-reversible analgesic activity versus control and Kyo. The most active compound Tyr-NsArg-Bzl was found to increase significantly the pain threshold versus native peptide Kyo on 15th minute. On 30th minute only Tyr-NsArg has analgesic effect compared to the control and Kyo. In time the analgesic effects of newly synthesized analogues quickly decreased and on the 45th minute they completely vanished.

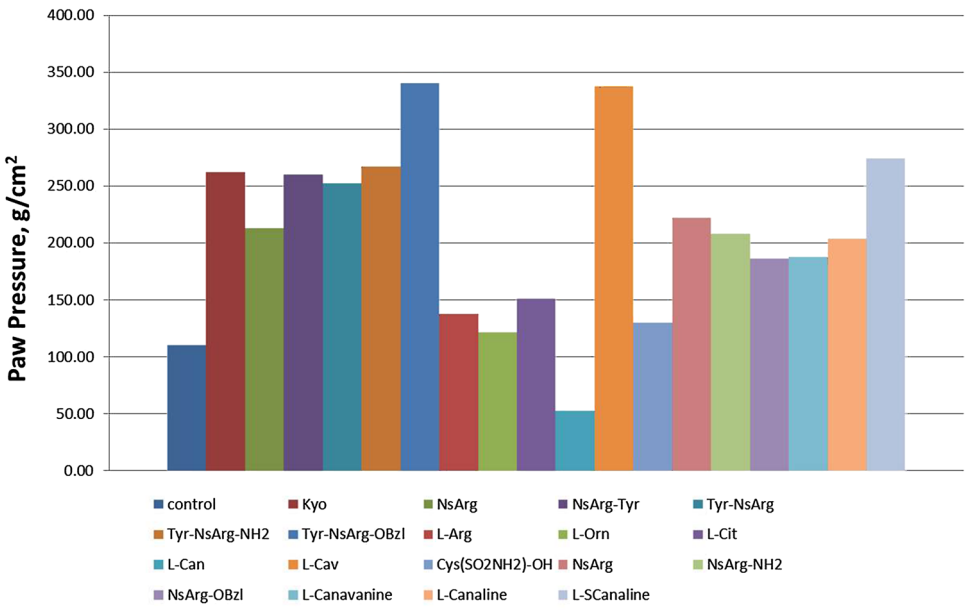

Fig. 3 Effects of kyotorphin analogues estimated by paw-pressure test. (L-Arg, L-Orn, L-Cit, L-Can and L-Cav—*i.c.v* administration, all at dose 20 μg/kg, Brakadanska *et al.,* 2001; Cys(O_2NH_2), NsArg, NsArg-NH_2, NsArg-OBzl, L-Cav and L-Can—*i.p.* administration, all at dose 1 mg/kg, and NsArg, NsArg-Tyr, Tyr-NsArg, Tyr-NsArg-NH_2, Tyr-NsArg-OBzl—*i.p.* administration, all at dose 5 mg/kg, Bocheva *et al.,* 2006). Data are presented as mean ± SEM

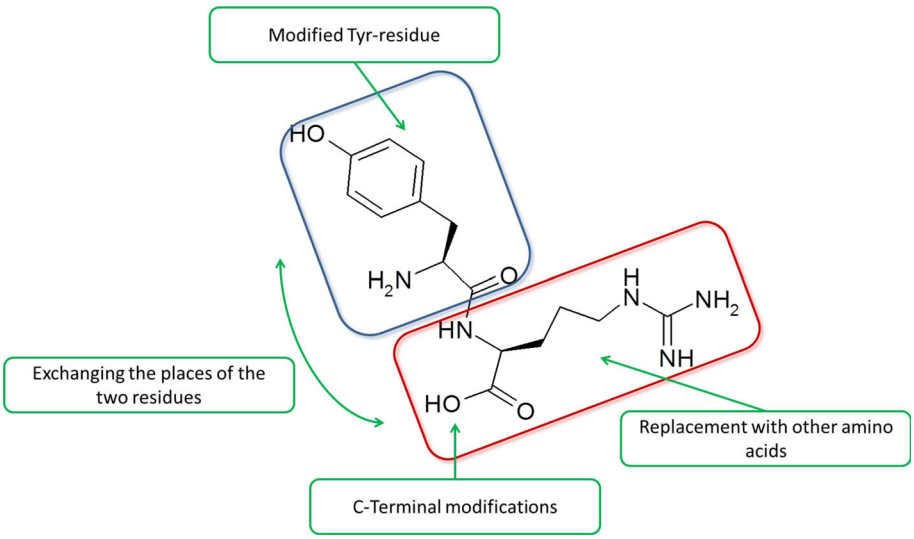

Fig. 4 Design of Kyo analogues

It is well documented that the mammalian brain contains all the urea cycle intermediates, whereas enzymes participating in the conversion of L-ornithine (L-Orn) to L-citrulline (L-Cit) are absent, resulting in an incomplete urea cycle (Garthwaite, 1991). On the other hand, in our previous in vivo experiments we demonstrated that L-Cav, L-Cit and L-Orn exerted antinociceptive effects (Brakadanska *et al.*, 2001; Bocheva *et al.*, 1996).

All this and the fact that L-norcanaline (NCan) and L-norcanavanine (NCav) are structural analogues of ornithine and canavanine, respectively, stimulated us to synthesize new analogues of kyotorphin which were expected to possess analgesic activity. Namely, NCan and NCav were introduced into the peptide chain of kyotorphin. Due to direct modification in both positions of the parent structure, four analogues with the following structural formulas were obtained: Tyr-NCav; Tyr-NCan; NCav-Tyr and NCan-Tyr.

The peptides were synthesized by a standard solution peptide synthesis. For the synthesis of reverse-kyotorphin analogues NCan-Tyr and NCav-Tyr condensation of Z-NCan, respectively Z-NCav and Tyr-OMe was applied, using method of mixed anhydrides (Fig. 5a).

The hydrolysis of methyl ester was done by 1 N NaOH in water: dioxane mixture (1:1), followed by acidolysis of Z-group with HBr/CH_3COOH. Final peptides were obtained in good yields after column purification (Silicagel 60, CH_3CN: H_2O, 4:1).

Modified in position 2 kyotorphins were synthesized using method of activated esters (Fig. 5b). The Z-Tyr-OSu

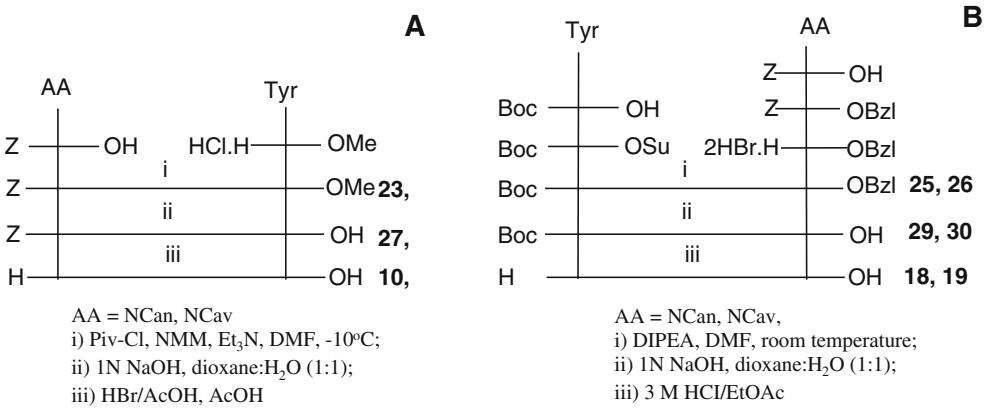

Fig. 5 Synthesis of NCan-Tyr and NCav-Tyr (**a**) and Tyr-NCan and Tyr-NCav (**b**)

reacted with benzyl esters of NCan or NCav prepared in advance. Reaction time was about 24 h fully protected peptides were obtained in 63 and 54 % yields respectively. Final peptides were obtained in good yields after column purification (Silica gel 60, CH_3CN: H_2O, 4:1).

Each peptide was analyzed for purity on high-performance liquid chromatography (HPLC) and characterized by ^{1}H- and ^{13}C-NMR, IR-spectrometry, and high-resolution mass spectra with a Fissons-Triple Quadrupol-ES mass spectrometer.

Conventional IR spectroscopy is not very informative for peptides but NMR spectra provide the most complete information on the structure of the obtained compounds. NCan is a structural analogue of the amino acid ornithine and NCav—of arginine, respectively. A comparison with published data in Biological Magnetic Resonance Data Bank (icnm.cerm.inifi.it) for natural amino acids was made. The shift of the protons of the δ-amino group of ornithine in ^{1}H-NMR is at 1.8 ppm. The effect of the bound oxygen atom is low and the shift of the bands of the analogues containing NCan in lower field is negligible. The chemical shift is at about 2 ppm.

However, the influence of the oxygen atom in the analogues containing NCav in their molecules is significant. In the spectrum of arginine the protons of the guanidinium group are shifted at 1.67–1.7 ppm, while the protons of the oxyguanidinium group are shifted to the lower field at about 8 ppm under the influence of the oxygen atom.

The chemical shift of the C-atoms of the guanidinium group of arginine is at 36.2 ppm in ^{13}C-NMR, while under the influence of oxygen atom this shift for the C-atom of the oxyguanidinium group is in lower filed at about 158 ppm.

Further, we tried to find relationship between structure and activity, and to predict the biological effect of the newly synthesized kyotorhin analogues **18** and **19**, using a docking sofware. In this regard, we collected data from paw-pressure tests for all compounds described above. Docking was carried out with GOLD 5.1 software. It uses genetic algorithm and considers full ligand conformational flexibility and partial protein flexibility. For docking studies the crystal structure of μ-opioid receptor, published in RCSB was used. It was published (Befort *et al.*, 1996) that the binding site for opioid receptors was defined as residues within 10 Å radius of aspartic acid of third trans membrane domain, which is involved in the most crucial interaction. In the case of μ-opioid receptor this is Asp147. GoldScore algorithm was used and Fitness scoring function was calculated for each ligand. All Fitness function's values are listed in the Table 1.

There is a good correlation between number of interactions between μ-opioid receptor and ligand, and its biological activity. The most potent compounds according to data in the Table 1, are Tyr-NsArgOBzl, Tyr-Cav, L-NAME, Tyr(Cl_2)-Cav. They interact with the receptor sterically, electrostatically, and by forming hydrogen bonds. In those cases number of interactions was more than 12. The ligands with the lowest potency [L-Orn, L-Cit, Cys(SO_2NH_2)] interacted weaker with receptor with not more than 6 interacting sites. Examples of interactions were shown on Fig. 6.

Exploring docking results, almost all studied ligands interacted with residue Asp147 from the μ-opioid receptor sequence. This residue was involved in forming hydrogen bonds with appropriate functional group of the ligand. The neighbor residue Tyr148 played important role in ligand-receptor interaction also, either by forming hydrogen bond with its OH group, or by π-π interactions with ligand.

The kyotorphin for example formed 6 hydrogen bonds with residues in the receptor pocket: its carboxylic group interacted with three residues Gln124, Asp147, and Tyr326; the free amino group interacted by hydrogen bonding with hydroxyl group of Tyr148; and the guanidinium group formed two hydrogen bonds with Gln124 (Fig. 7a). One of the newly synthesized kyotorphin analogues (NCav-Tyr) interacted with

Table 1 Values from paw-pressure test from in vivo studies and fitness function from docking studies for all studied compounds

Number	Compounds	PP, g cm^2	Fitness
1	Cys(SO_2NH_2)	130	41.37
2	D-Kyo	241[a]	64.72
3	Kyo	265[b]	69.38
4	L-Can	204	44.32
5	L-Cav	188	54.51
6	L-Cit	151	45.71
7	L-NAME	283[b]	54.00
8	L-NCan	274	36.19
9	L-Orn	121	40.56
10	NCan-Tyr	235	57.92
11	NCav-Tyr	295	64.77
12	NsArg	222[c]	49.42
13	$NsArgNH_2$	208	52.02
14	NsArgOBzl	186	57.51
15	NsArg-Tyr	260[b]	61.02
16	Tyr-Cav	308[b]	65.23
17	Tyr(Cl_2)-Cav	280[b]	68.83
18	Tyr-NCan	220	55.49
19	Tyr-NCav	280	63.21
20	Tyr-NsArg	252[c]	66.67
21	Tyr-$NsArgNH_2$	267[c]	65.83
22	Tyr-NsArgOBzl	340[c]	77.15

In red are presented predicted values of threshold of the newly synthesized kyotorphin analogues

[a] Results of previous research (Dzambazova *et al.*, 2011)

[b] Results of previous research (Dzambazova-Maximova *et al.*, 2003)

[c] Results of our previous research (Bocheva *et al.*, 2006)

μ-opioid receptor by forming 7 hydrogen bonds. The oxyguanidinium group formed two hydrogen bonds with Tyr148, the free amino group was connected with Asp147, the free carboxylic group interacted by forming 3 hydrogen bonds with Asp147, Tyr326, and Gln124, respectively and the hydroxyl group was hydrogen bonded with Cys217 residue (Fig. 7b).

Significant relationship was found between external energy of the bonded ligands and their biological effect. In this context, as less is this energy, as higher is the biological effect of investigated compound.

Correlations of docking data and results from the in vivo experiments were performed with GraphPad Prism 3.0. The pain threshold in g cm^2 was compared with the results from docking (Fitness scoring function) in order to find correlation. Good positive Pearson's correlation was obtained between Fitness scoring function from GOLD docking procedure with threshold at 15th minute of paw-pressure test (Fig. 8). As higher is a value for Fitness scoring function as higher is biological effect of the investigated compound. Value for Fitness scoring function could be easily obtained from docking results for any compound with μ-opioid receptor. Using this model it is simply to calculate the values of paw-pressure threshold and predict the biological effect.

As well as docking was performed with the μ-opioid receptor structure, and there is correlation between in vivo results and Fitness function, assumption made from us before could be true. In other words, if the kyotorphin receptor exist its binding pocket, is the similar as in the μ-opioid receptor.

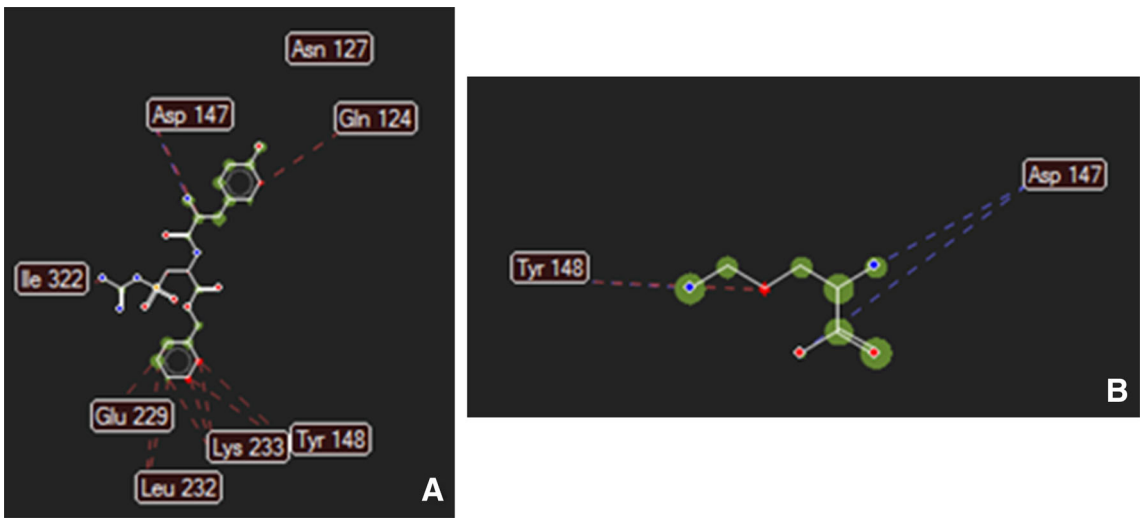

Fig. 6 Interactions in the binding pocket of μ-opioid receptor of Tyr-NsArg-OBzl (**a**) and L-Orn (**b**). Colors in the pictures *red*—steric and electrostatic interactions, *blue*—hydrogen bonds (Color figure online)

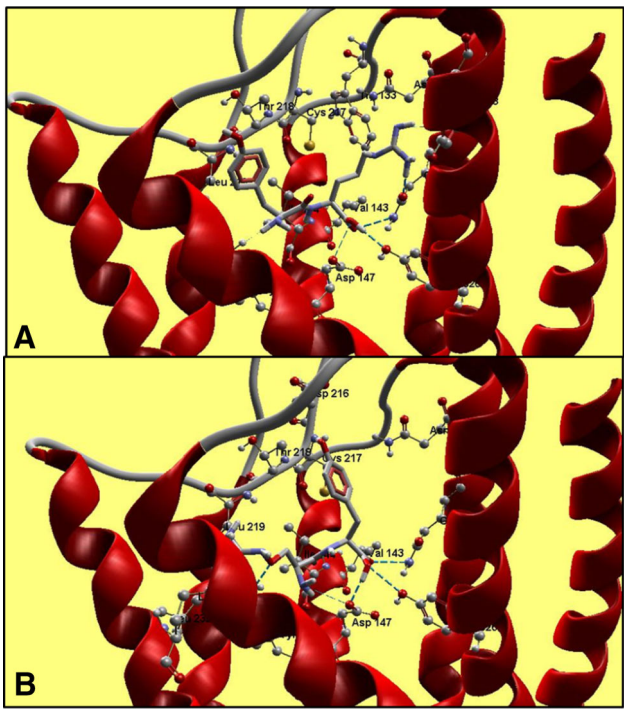

Fig. 7 Hydrogen bond formed in binding pocket of μ-opioid receptor with kyotorphin (**a**) and NCav-Tyr (**b**)

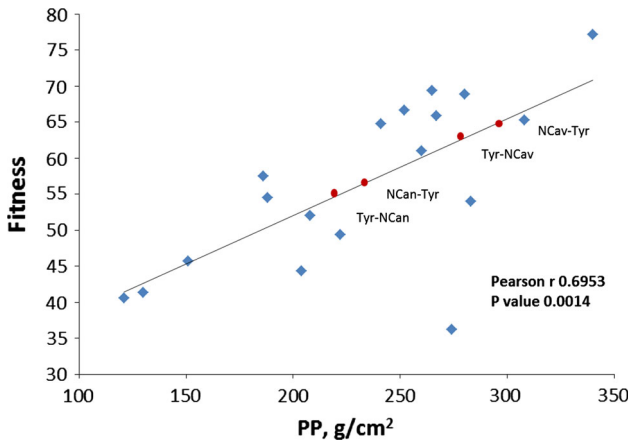

Fig. 8 Correlation between Fitness function of studied compounds and data from in vivo tests

Assuming that there is Pearson's correlation with linear regression of the docking results and in vivo biological activity, we can suggest that newly synthesized analogues will have the following activity, as shown on Fig. 8 (with red dots are presented predicted ranks of the compounds). These results elucidated the ligand-receptor interactions and could be helpful for the discovery of new active analogs of KTP.

Conclusion

In summary, four new analogues of kyotorphin containing NCan and NCav were synthesized using well-known methods of peptide synthesis in solution. We have demonstrated that arginine mimetics, as well as amino acids from urea cycle exerted strong naloxone-reversible antinociceptive effects in in vivo test.

The molecular docking procedure was applied to determine the possible interactions for our ligands and the receptor. From docking studies we could conclude that there is evidence to assume that even kyotorphin receptor exists its binding pocket, is not very different from those of μ-opioid receptor. By means of the docking studies we could predict biological effects of newly synthesized compounds, and we could propose structures with higher biological activity.

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Conflict of interest The authors declare that they have no conflict of interest.

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