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Kyotorphin analogues – transport and enzymatic stability studies with computational methods

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Abstract
Peptide exhibiting a biological effect once inside the body undergoes multiple biotransformations leading to their inactivation and degradation. An effective way for its use is the introduction into the body simultaneously with peptidase inhibitors. Another viable approach is the development of peptide mimetics that exhibit the same or increased biological effect, but increased stability.
Here we present the docking studies of kyotorphin analogues with already known in vivo biological effect regarding aminopeptidases and oligopeptide transporters. The objective was to determine whether these analogues exert their pronged effect due to their stability to the peptidases action and also whether they can be transported by the transmembrane transporters which also influence their effects.
The docking results showed that kyotorphin analogues can not be hydrolyzed by aminopeptidase N and only three of them can be transported through the cell membrane. From these studies it was concluded that the tested analogues are effective, stable, and do not affect overall metabolism, which makes them suitable for the treatment of pain.

Keywords: Kyotorphin, docking, aminopeptidase N, ptoton-dependent oligopeptide transporter.

1. Introduction
Neuropeptides, including Met-enkephalin, Leu-enkephalin, dynorphin, kyotorphin (Kyo), endomorphins and many others, are part of the family of the endogenous opioid peptides and they act directly to the opioid receptors leading antinociceptive effects [1-3]. The endogenous opioid peptides act as "natural analgesics". They have a structural relationship because many of them have l-tyrosine as N-terminal residue, which is very important for the activity of the peptide [4].
Kyotorphin (Kyo) is an endogenous Met-enkephalin releasing antinociceptive dipeptide (l-tyrosyl-l-arginine). It is synthetized in mammal by kyotorphin synthetase from l-tyrosine and l-arginine. But the action of Kyo decreases quickly because the peptide is rapidly degraded by bestatin-dependent aminopeptidase. This enzyme catalyzes also degradation of Met- and Leu-enkephalin [5, 6]. Later, a new synaptic membrane-bound amino peptidase was identified. It was suggested that this enzyme is kyotorphin-hydrolyzing peptidase [7]. This enzyme was isolated and many biochemical studies were performed with it but its crystal structure is not identified yet. Most of the extracellular peptide-degrading enzymes are membrane-bound exo- and endopeptidases and aminopeptidase N is one of these crucial enzymes. APN is a transmembrane protease present in a wide range of human tissues and cell types (endothelial, epithelial, fibroblast, leukocyte), which is able to modulate bioactive peptide responses and to influence immune functions. APN is responsible for the degradation of several biologically active peptide substrates including Enkaphalins, neurokinin A and β-EP [8-11].
However, there are also peptide transporters in the brain, for example, members of the proton-coupled oligopeptide transporters (POT) family [12, 13]. Members of this family are widespread in living organisms. They transport the short peptides and amino acids through cell membranes. This is realized due to and inwardly directed proton (H^+) gradient. Members of the human family, Pept1 and Pept2, are responsible for the uptake of di- and tripeptides in the small intestine and the kidney, respectively. They are also involved in the major metabolic pathway for absorption of nitrogen required for the body normal functioning, but also to absorb orally administered drugs, such as β-lactam antibiotics.

Since it is relatively difficult to produce large quantities of human membrane proteins, mainy studies are conducted with their bacterial homologues. Bioinformatics searches show that the transporter from *Shewanella oneidensis* has a high amino acid sequence similarity (~30 %) to the human protein when compared to many other bacterial transporters.

Recent study shows that when these transporters are not present analgesic action of Kyo increases [14]. We present here computational approach for explanation of the action of kyotorphin analogues, previously synthesized and biologically tested [15, 16] to aminopeptidase N (APN) and POT. This study was performed in order to explain their prolonged analgesic effects and to try to make some prediction about the action of newly synthesized analogues.

2. Methods

2.1 Enzyme, transporter and kyotorphin analogues.

Crystal structures are obtained from RCSB: PDB id 4fyr for APN and 2xut for POT, respectively. Kyotorphin analogues (Table 1) were previously synthetized and biologically tested [15-18].

2.2 Computational tools.

In order to perform computational studies, the following software was used in the present work: compound preparation was performed with Avogadro 1.1.0 (http://avogadro.cc/wiki/Main_Page)docking studies were performed by using PatchDock Server [19, 20]; for the generation figures and explanation of the interactions after docking the Molegro Molecular Viewer(MMV) [21] was applied.

2.3 Docking of kyotorphin analogues.

All 12 dipeptides: kyotorphin and its analogues were modeled with Avogadro 1.1.0 and protonated at physiological pH. Docking was performed on PatchDock Server; it uses a geometry-based molecular docking algorithm. The active sites of APN[22] and POT[23] were defined according to literature.

Table 1: Kyotorphin analogues used in our study.

Peptide	Structure	Reference
DKyo	l-tyrosyl-d-arginine	(17)
Kyo	l-tyrosyl-l-arginine	
NsArg-Tyr	l-norsulgoarginyl-l-tyrosin	(15; 16)
Tyr(Cl_2)-Cav	l-dichlorotyrosyl-l-canavanine	(18)
Tyr-Cav	l-tyrosyl-l-canavanine	(18)
Tyr-NsArg	l-tyrosyl-l-norsulfoarginine	(15; 16)
Tyr-NsArg-NH_2	l-tyrosyl-lnorsulfoarginine amide	(15; 16)
Tyr-NsArg-OBzl	l-tyrosyl-l-norsulfoarginine benzyl ester	(15; 16)
NCan-Tyr	l-norcanalyl-l-tyrosine	
NCav-Tyr	l-norcanavanyl-l-tyrosine	
Tyr-NCan	l-tyrosyl-l-norcanaline	
Tyr-NCav	l-tyrosyl-lnorcanavanine	

3. Results and discussion

3.1 Docking of APN

As well as software cannot give as information about enzyme-catalyzed reactions we defined the criteria by which the results will be evaluated. It is known [22] that the most important residues for the substrate recognition are Glu355 and Glu411 from the enzyme sequence. Furthermore, enzyme-catalyzed hydrolysis can occur only if C=O group from the first peptide bond is close enough to Zn^{2+}. In figure 1 is presented the mechanism of action of APN. And distances required between key residues of enzyme sequence and important parts of the substrate molecule.

Analyzing results from the docking for Kyo it is clear that there are all prerequisites for the realization of the enzyme-catalyzed reaction. Data for the distances are Glu355 / N – 2.65 Å, Glu411 / N – 3.64 Å, and Zn^{2+} / O – 2.30 Å, respectively. In the table 2 the distances between key residues and Zn2+ and important part from the molecules of kyotorphin analogues are presented. Data are for the best arranged with respect the active site pose of the corresponding analogue.

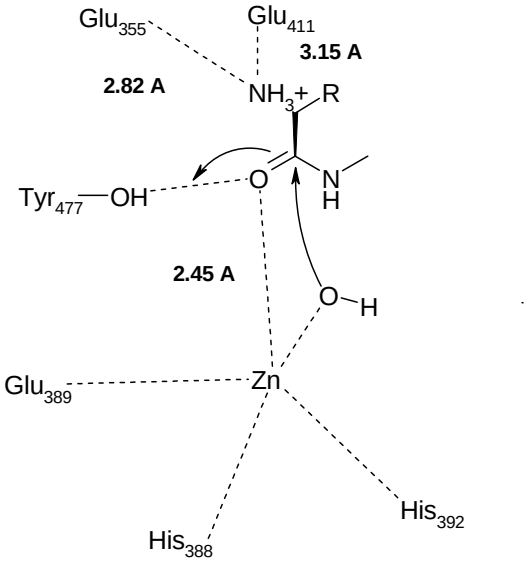

Fig 1: Schematic presentation of mechanism of action of APN. The distances are according to crystal structure PDB id: 4fys.

The table shows that there is no analogue which binds in a suitable manner with the active site of the enzyme. The distances between the residues of glutamic acid, Glu355 and Glu411, responsible for recognition of the terminal amino group and the nitrogen from this group is too large. Hence, the substrate molecule cannot bind well to the active site of the enzyme and therefore C=O group may not occupy an appropriate position relative to Zn^{2+}. This makes impossible the realization of the enzyme-catalyzed reaction.

According to these data Kyo analogues could not act as substrates for APN. From literature[22] it is known that bestatin, a peptidase inhibitor, interacts directly with Zn^{2+}, thus blocking the enzyme action. All tested analogues bind far from Zn^{2+}, as shown by the results in the table 2. Zn^{2+} is free and can participate in the reaction. However, analogues themselves could not be hydrolyzed by the enzyme, since there are no spatial prerequisites for this. But there presence in the body could prevent partially the hydrolysis of various biological peptides, natural substrates of the enzyme. This is due to the fact that kyotorphin analogues bund in the active site, thus preventing its access to other molecules.

Table 2: Distances between key residues in APN sequence and important points of substrate molecule and total energies of enzyme-substrate complexes

Peptide	Distances in Å			Total energy, kJ/mol
	Zn^{2+} - O	Glu355 - N	Glu411 - N	
Angiotensin IV *	2.458	2.82	3.15	-132.061
DKyo	6.058	6.707	5.108	-42.215
Kyo	2.30	2.653	3.17	-61.23
NsArg-Tyr	8.48	10.734	10.629	-55.865
Tyr(Cl_2)-Cav	4.199	6.2995	5.444	-31.216
Tyr-Cav	9.081	12.588	10.797	-54.464
Tyr-NsArg	7.988	7.115	6.161	-53.037
Tyr-NsArg-NH_2	9.468	11.333	10.108	-40.371
Tyr-NsArg-OBzl	11.117	8.749	8.802	21.965
NCan-Tyr	8.782	8.771	9.043	-68.487
NCav-Tyr	9.197	8.899	8.675	-69.426
Tyr-NCan	5.335	6.702	5.665	-57.659
Tyr-NCav	7.632	9.499	9.456	-69.652

* These data was obtained from crystal structure, PDB id 4fys.

From the total energies of the enzyme-substrate complexes we can make similar conclusions. Comparing these energies it is seen that all analogues have very high values of total energies. In the case of benzyl ester of kyotorphin containing norsulfoarginine in the second position the energy is even positive. This is most likely due to the fact that there is a strong steric hindrance in the formation of enzyme-substrate complex because of benzyl moiety. Substrate molecule itself occupies energetically unfavorable conformation in which there is a tension in the molecule, and its total energy is positive (the energy of torsional strain is 11.769 kJ/mol and the energy of steric interaction is 63.594 kJ/mol). From an energetic point of view, such an enzyme-substrate complex is unlikely to occur.

The total energies of complexes of APN with analogues of kyotorphin with NCav in the first and second position and NCan in the first position have values less than that of the complex APN-Kyo. This occurs due to the small size of the molecules, the lower basicity of the side chains of NCan and NCav, and hence weaker repulsive forces with the enzyme surface.

These three analogues sufficiently strongly to the enzyme, and they would play role of its inhibitors. Since they cannot be hydrolyzed they will remain attached to the enzyme inhibiting the binding of other substrates, and thus inhibiting the enzyme action.

It is known that the effect of the biologically active peptides is highly influenced by the action of peptidases. The strong effect of kyotorphin analogues can be explained by the failure of peptidases to recognize them as substrates. Hence, they are more stable in biological conditions and can exhibit their effects more prolonged.

Kyo binds to the kyotorphin receptor whose active site has a structure analogous to that of μ-receptor. Kyo degrades in biological conditions rapidly under the action of peptidases and in the time its effect rapidly decreases.

Kyotorphin analogues (our previous study, article in press) bind well to μ-opioid receptor which determines their strong analgesic effect. The duration of this effect can be explained in particular by their stability towards peptidases.

The four kyotorphin analogues containing NCan and NCav in the first and second position would have again prolonged effect according to data presented.

All analogues are stable with respect to APN, while preventing its effects towards other biological peptides. Since their binding energies are not very low, they can be regarded as competitive inhibitors of APN. High doses of biological substrates would get shifted them from the active site and the catalytic reaction would occur. They will exhibit their biological effect; they will be sufficiently stable, while it has no effect on the metabolism of other classes of biological molecules.

3.2 Docking POT

The central cavity of the transporter is 13 x 12 x 11 Å. These dimentions are sufficient for the binding of the di- and tripeptides, while with larger tetrapeptides steric

interferences will appear. The binding site of the transporter iis formed by positively charged residues Arg25, Arg32, and Lys127 from the N-terminal side. The C-terminal side is at a distance of approximately 13 Å from Lys 127. Two strictly conserved residues, Glu413 and Ser423, are involved in interactions, and they are located relatively closed to Tyr154.

Furthermore, it is found that exists so-called intracellular gate formed by the interactions of side chains of the amino acid residues located in the helices on the both side of the cavity. These are the interactions between residues Leu427 and Tyr154 and Phe150. These residues are conserved in POT family (motif FYxxINxG), and it is assumed to play a role in regulating the exit of the peptides from the central cavity in the cytoplasm.

Although, the transporter has been studied in depth using the biochemical and biophysical methods, little is known on the structure of its different states, as well as the mechanism of transport of the small peptides. This is why we present here our assumptions based on the obtained docking results.

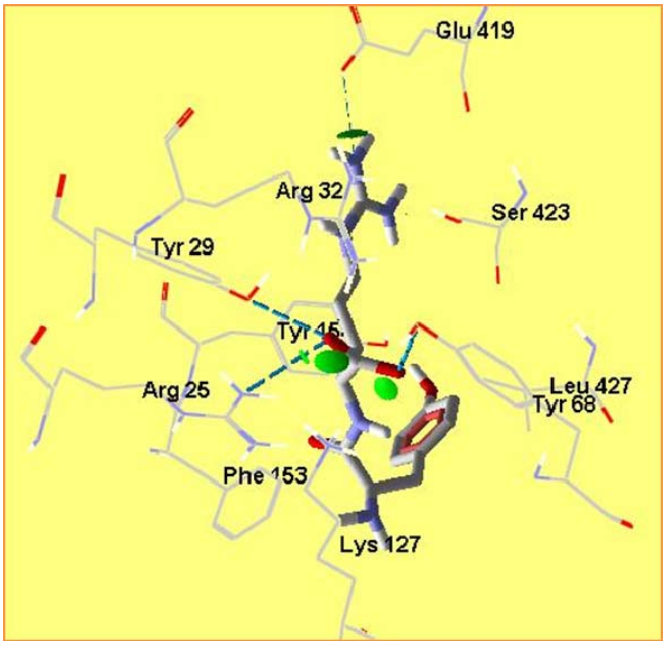

Fig 2: Interactions in the binding site of POT with kyotorphin.

As seen from the figure 2 particularly important requirements for identification of the dipeptide are interactions of the free carboxyl group with Arg25, Arg32, Tyr68, and Tyr29. On the other hand to accomplish a successful transport is necessary to be opened the so-called intracellular gate. In the case of Kyo it can happen, as it strongly interacts electrostatically with the side chain of Lys127. This makes impossible Lys127 to interact with Tyr154 and Phe150 and to limit the Kyo access to cytoplasm. Similarly strong interactions with Lys127 are observed in the cases of NCav-Tyr, NsArg-Tyr, and Tyr(Cl_2)-Cav. The energies of these interactions (Table 3) are low enough to allow them to take place. Free carboxyl group of NsArg-Tyr and Tyt(Cl_2)-Cav again interacts with amino group of the side chain of Lys127. There is recognition of the dipeptides by the transporter which would help their transport through the membrane. In the case of NCav-Tyr phenolic hydroxyl group of tyrosine residue is interacted with the amino group of Lys127. Although there is no interaction between carboxyl and amino group, this interaction is strong enough, and could lead to the opening of the gate and passage of this analogue in the cell.

When connecting the Kyo to the binding site of POT considerable importance has a guanidine group. It binds electrostatically with Glu419. All analogues excluding DKyo contain an arginine mimetic with reduced basicity of the side chain. This is why they can not interact with the carboxyl group of the side chain of Glu419.

The data from this analysis (Table 3) show that complexes of Kyo analogues excluding complexes POT/Tyr-NCan, POT/Tyr-NsArg, and POT/Tyr-NsArg-NH_2 are energetically favorable, i.e. these analogues will interact with transporter. But they will pass or not into the cell will depend not only on the ability to bind as well as on the mode of binding.

The total energy of the complex POT/DKyo is the lowest in the test series, i. e. this complex will be formed easily and will be stable enough. But the fact that DKyo binds differently to POT than Kyo makes its passage into the cell with the aid of the transporter impossible. DKyo, Tyr-Cav, Tyr-NsArg, NCan-Tyr and Tyr-NCav will block in a certain extent POT, since they are associated strongly enough with it, but can not pass through it. However, the energies of complexes with POT are of the order of the energy of the complex POT/Kyo, which means that higher concentrations of small natural peptides would shift them from POT.

This is another proof that Kyo analogues particularly useful in the treatment of pain. On one hand they exhibit strong analgesic effect, but do not affect key metabolic processes.

The conclusion that could be drawn for the analogues with respect to POT is that three of them,NCav-Tyr, NsArg-Tyr, and Tyr(Cl_2)-Cav, will pass into the cell with a help of POT. This will lead to their more rapid removal and therefore will affect their effect on μ-opioid receptor. The other analogues can not be transported by POT and therefore will be available for a prolonged action. Three of the newly synthesized Kyo analogues, Tyr-Cav, NCan-Tyr and Tyr-NCav, may be expected that will exhibit a prolonged analgesic effect as they will not be able to pass quickly the cell membrane.

Table 3: Total energy of the complex POT-dipeptide and energy of interaction of dipeptide with Lys127.

Peptide	Total energy, kJ/mol	Energy of interaction with NH_2 group of Lys127, kJ/mol
DKyo	-75.208	-
Kyo	-63.241	-10.0083
NsArg-Tyr	-70.387	-10.5003
Tyr(Cl_2)-Cav	-56.503	-15.8424
Tyr-Cav	-61.929	-
Tyr-NsArg	-44.628	-
Tyr-NsArg-NH_2	-48.633	-
Tyr-NsArg-OBzl	-51.650	-
NCan-Tyr	-73.739	-
NCav-Tyr	-70.956	-11.988
Tyr-NCan	-40.855	-
Tyr-NCav	-60.506	-

4. Conclusions

All investigated kyotorphin analogues have a prolonged action compared to those of kyotorphin. This effect can be successfully explained with their stability with respect to peptidases and impossibility to be transported through cell membrane by oligopeptide transporters.

These kyotorphin analogues can be used for pain treatment in vivo as they are effective, stable and do not affect the metabolism of other natural substances.

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