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ORIGINAL ARTICLE

1-Substituted sialorphin analogues—synthesis, molecular modelling and in vitro effect on enkephalins degradation by NEP

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Abstract
Rat sialorphin (Gln-His-Asn-Pro-Arg) is a natural blocker of neprilysin (NEP) that belongs to the family of endogenous opioid peptide-degrading enzymes. Studies have confirmed the efficiency of sialorphin in blocking the activity of NEP, both in vitro and in vivo. It has been demonstrated that this inhibitor has a strong analgesic, anti-inflammatory, immunological and metabolic effect either directly or indirectly by affecting the level of Met/Leu-enkephalins. In this work, sialorphin and their 12 analogues were synthesised using the solid-phase method. The effect of the peptides on the degradation of Met-enkephalin by NEP and metabolic degradation in human plasma was investigated in vitro. We show that the change in the *N*-terminal amino acid configuration from L to D in almost all peptides, except D-Arg-His-Asn-Pro-Arg (peptide XI), led to the abolition of their inhibitory activity. With molecular modelling technique we explained the structural properties of the L and D-arginine located on the *N*-terminal part of the peptide. The detailed analysis of the protein binding pocket allowed us to explain why D-arginine is so unique among all D residues. Peptide XI showed the highest stability among the tested peptides in human plasma. For instance sialorphin after a 2-hour incubation in human plasma was almost completely decomposed, while the level of peptide XI dropped to 45% after 48 h under these conditions.

Keywords Enkephalins · Sialorphin · Neutral endopeptidase · Peptides synthesis · Molecular modelling · Structure–activity relationships

Abbreviations

ACE	Angiotensin-converting enzyme
Boc	*Tert*-butyloxycarbonyl
DCM	Dichloromethane
DIC	*N*,*N*′-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
Fmoc	9-Fluorenylmethoxycarbonyl
HOBt	1-Hydroxybenzotriazole
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NEP	Neutral endopeptidase
Pbf	2,2,4,6,7-Pentamethyl-dihydrobenzofuran-5-sulfonyl residue
RP-HPLC	Reversed-phase high-performance liquid chromatography
SPPS	Solid-phase peptide synthesis
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
Trt	Trityl

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Introduction

Currently there are three types of opioid receptors in mammalian tissues, namely mu, kappa, and delta ones (MOR, KOR, and DOR, respectively). Classically, they have been known to be involved in pain signaling pathways and their exogenous ligands have been used in the treatment of

inflammatory and cancer pain. More recently, as our knowledge on opioid receptors expanded, their involvement in several other physiological and pathophysiological processes, such as gastrointestinal (Gl) tract and immune system functions paved the way to their use for targeting conditions other than pain (Mosińska et al. 2016).

Endogenous opioid receptor ligands Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) share similar biological properties with their exogenous counterparts including antinociception and the regulation of GI motility, secretion, proliferation, and immune functions (Mosińska et al. 2016). However, they are rapidly destroyed and their pharmacological action is dissipated. Degradation of enkephalins has been studied in vitro (Jakas and Horvat 2004; Rošcic et al. 2008) and in vivo (Roques et al. 1993; Sokolov et al. 2004) and dependence on both species and tissue type were found (Bolacchi et al.1995). For instance, the half-lives of Met-enkephalin and Leu-enkephalin in the human serum in vitro study were 12.2 min (Jakas and Horvat 2004) and 14.8 min (Rošcic et al. 2008), respectively, while the disappearance of enkephalins when incubated with rat brain membranes or plasma took place within 1 min (Roques et al. 1993). In the human tissues, several enzymes hydrolyzing different peptide bonds in enkephalin molecules were found (Marini et al. 1990). These include neutral endopeptidase (NEP, EC 3.4.21.11), dipeptidyl peptidase (DPP EC 3.4.14.4), aminopeptidase *N* (APN, EC 3.4.11.2) and angiotensin-converting enzyme (ACE). Importantly, the peptidases involved in the degradation of enkephalins in human plasma (soluble peptidases) are not necessarily similar from those involved in tissue synaptic spaces. Nevertheless, in vivo as in vitro, APN cleaves the enkephalins at the *N*-terminus to give the main inactive metabolites: Gly-Gly-Phe-Met and Tyr. Tyr-Gly-Gly and Phe-Met are the two others major inactive metabolites linked to NEP endopeptidase activity. While, DPP III activity gives two minor metabolites: Tyr-Gly and Gly-Phe-Met (Roques et al. 1993).

In our previous study, we demonstrated that sialorphin, a physiological inhibitor of Zn-dependent metallo-ectopeptidase NEP, could increase the half-life of enkephalins in vitro (Rougeot et al. 2003; Kamysz et al. 2016). This exocrine and endocrine signalling pentapeptide (Gln-His-Asn-Pro-Arg) is synthesised predominantly in the submandibular gland and prostate of adult rats in response to androgen steroids and is released locally and systemically in response to stress (Rougeot et al. 1997, 2003; Messaoudi et al. 2004; Davies et al. 2007). Sialorphin occurs in rat urine, as well as in the mammary glands and milk and placenta of female rats (Dufour et al. 2013; Rougeot 2004). This peptide displays a potent, naloxone-sensitive antinociceptive effect in behavioural models of acute pain (Rougeot et al. 2003) plays an important role in the control of social behaviour, enhances sexual behaviour and erectile function in male rats (Messaoudi et al. 2004; Davies et al. 2007). Sialorphin is also believed to be involved in the regulation of systemic mineral ion homeostasis (Rougeot et al. 1997, 2003) As reported recently, sialorphin attenuated acute, semichronic as well as relapsing 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice after systemic administration (Kamysz et al. 2016; Sałaga et al. 2017) and displays antinociceptive efficiency in the mouse model of visceral pain induced by colorectal distension (Fabisiak et al. 2018).

In the present study, we characterized in vitro the effect of 1-substituted sialorphin on the degradation of Met-enkephalin by NEP and their stability in human plasma. We also used molecular modelling technique to explain the influence of the change of configuration from L to D amino acid residues in position 1 in sialorphin molecule on the ligand–enzyme interactions.

Materials and methods

Peptides synthesis

All the peptides were synthesised manually via the soild-phase method using Fmoc (9-fluorenylmethoxycarbonyl) chemistry. A 2-chlorotrityl chloride resin (loading 0.3–0.9 mmol/g, 1% DVB, 200–400 mesh, Orpegen Peptide Chemicals GmbH, Heidelberg, Germany) was used as a solid support. The α-amino groups of amino acids were Fmoc protected. The amino acid side chain protecting groups were: Boc for Lys, D-Lys, Val, D-Val, Orn and D-Orn; Pbf for Arg and D-Arg; and Trt for Gln, Ile, D-Ile, His and Asn. *N*-α-protected amino acids, and the reagents used for the solid-phase synthesis were purchased from Iris Biotech GmbH (Marktredwitz, Germany). The first amino acid was bound to the resin according to Barlos et al. (1991) with a loading dose of 0.7 mmol/g. Peptide chains were elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with a 20% piperidine solution in *N,N*-dimethylformamide (DMF), whereas chain elongation was achieved with standard *N,N′*-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) chemistry using three equivalents of protected amino acid derivatives. The completeness of each coupling step was monitored by the chloranil test (Vojkovsky 1995). After completing the syntheses, the peptides were cleaved from the resin simultaneously with the side chain deprotecting groups in a single-step procedure using a trifluoroacetic acid (TFA):triisopropylsilane (TIS):H_2O (95:2.5:2.5, v/v/v) mixture. The peptides were precipitated with ice-cold ether and lyophilised.

The crude peptides were purified using HPLC on a Beckman controlled by Lp-Chrom system with an RP Kromasil-100, C8, 5 µm column (8 × 250 mm; Knauer, Germany) and a UV detector. The solvent systems were 0.1%

TFA in water (A) and 0.1% TFA in acetonitrile (B) and a linear gradient of 2–40% B over 30 min at a flow rate of 10 mL/min and the eluent was monitored at 214 nm. The purity of the synthesised peptides was checked on an RP Kromasil-100, C8, 5 µm column (4.6 × 250 mm; Knauer, Germany). The solvent system was as aforementioned. Fractions containing the pure peptides were pooled and lyophilised. Matrix-assisted laser desorption/ionization mass spectrometry (a Biflex III MALDI TOF instrument, Bruker Daltonics, Germany) was used to confirm identity of the pure products.

Determination of Met-enkephalin degradation rates

The degradation studies were performed using the NEP enzyme extracted from porcine kidney, acquired from Merck (Warsaw, Poland). Determination of Met-enkephalin degradation rates was performed, as described previously (Sobocińska et al. 2018). Samples consisting of the enzyme (5.687 nM), Met-enkephalin (0.0413 mM) and inhibitor (0.156 mM) were incubated over 0, 30, 60, 90 and 120 min at 37 °C in a final volume of 300 µL. The reaction was stopped at the required time by placing the tube on ice and acidifying with 30 µL of 1 M aqueous HCl solution. The aliquots were centrifuged at 14,500 rpm for 20 min. The supernatants were filtered and analysed by RP-HPLC. For the determination of inhibitory activity of peptides I–XIII against NEP at least three independent experiments were carried out.

Stability sialorphin and peptides II, XI in human plasma

Plasma-stability assays were performed by applying a modified procedure described by Seebach et al. (2011) and Roščić et al. (2008). Each peptide solution was prepared by dissolving 1 mg of a peptide in 1 mL of PBS buffer (pH 7.42). The samples containing the peptide (200 µL) and a freshly refrozen plasma (50 µL) were incubated at 37 °C. They were analyzed after 0, 2, 24 h (sialorphin (peptide I) and peptide II) and 0, 2, 24 and 48 h (peptide XI). At each time interval 40 µL of the mixture was taken and deproteinised by addition of a 48% aqueous TFA (8 µL) and PBS buffer (112 µL). The aliquots were centrifuged at 14,500 rpm for 5 min. The supernatants were filtered over Millipore Millex-GV syringe filters with a 0.22 µm pore size PVDF membrane (Millipore) and analysed with RP-HPLC on a Phenomenex Gemini-NX C18 5 µm column (4.6 mm × 150 mm) using the solvent system of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) and linear gradients of 1–30% (B) over 20 min (peptides I and XI) and 2–60% (B) over 20 min (peptide II). To assess the stability at each time interval, the area of the peptide peak from the chromatogram was calculated and expressed as the percentage of the area of the peak recorded just after mixing a peptide with serum (0 hour). The assay was performed at least in triplicate for each peptide.

Molecular modelling

The models of human neprilysin complexed with peptides X and XI (Arg-His-Asn-Pro-Arg, with L and D arginine in position 1) were constructed based on the previously described model of the NEP–sialorphin complex (Sobocińska et al. 2018). In short, the model is based on a human NEP structure (PDB ID: 2QPJ) with an inhibitor (Oefner et al. 2007). The inhibitor, as present in the 2QPJ structure, was computer-mutated to sialorphin. Subsequently, in this study the residue in position 1 (glutamine) was replaced by arginine in L and D configuration (Fig. 1). In the next step, Ψ angle of the [D-Arg^1] was rotated to fit the NEP binding grove. Both newly obtained complexes were optimized in AMBER force field (Kamysz et al. 2016; Pearlman et al. 1995; Case et al. 2005). The protein peptide interface was analysed using a RasMol AB program (Pikora and Giełdoń 2015) and visualization was made by PyMOL program (DeLano 2002). Electrostatic surface was created using ABPS PyMOL plugin (Jurrus et al. 2018).

Statistics

Statistical analysis was performed using the Prism 5.0 (GraphPad Software Inc., CA, USA) software. The data are expressed as mean ± SEM. The Student *t* test followed by Newman–Keuls posthoc test were used for the analysis. p values < 0.05 were considered as statistically significant.

Results and discussion

Peptide synthesis and purification

Sialorphin and its analogues (II–XII) modified in the *N*-terminal part of the molecule by exchanging Gln^1 for Ile^1, Val^1, Lys^1, Leu^1, Arg^1, Orn^1 with configuration L and D were synthesised using the solid-phase method (Chan and White 2000). The purity of the peptides after RP HPLC purification was higher than 98%. The identity of all peptides was confirmed by mass spectrometry MALDI-TOF and their calculated pseudomolecular ions values were in agreement with the theoretical ones. The sequences and physicochemical characteristics of peptides I–XIII are shown in Table 1.

Effect of sialorphin and its analogues on degradation of Met-enkephalin by NEP

Degradation rates (k) and half-lives ($t_{1/2}$) of Met-enkephalin incubated with NEP alone and in the presence of peptides

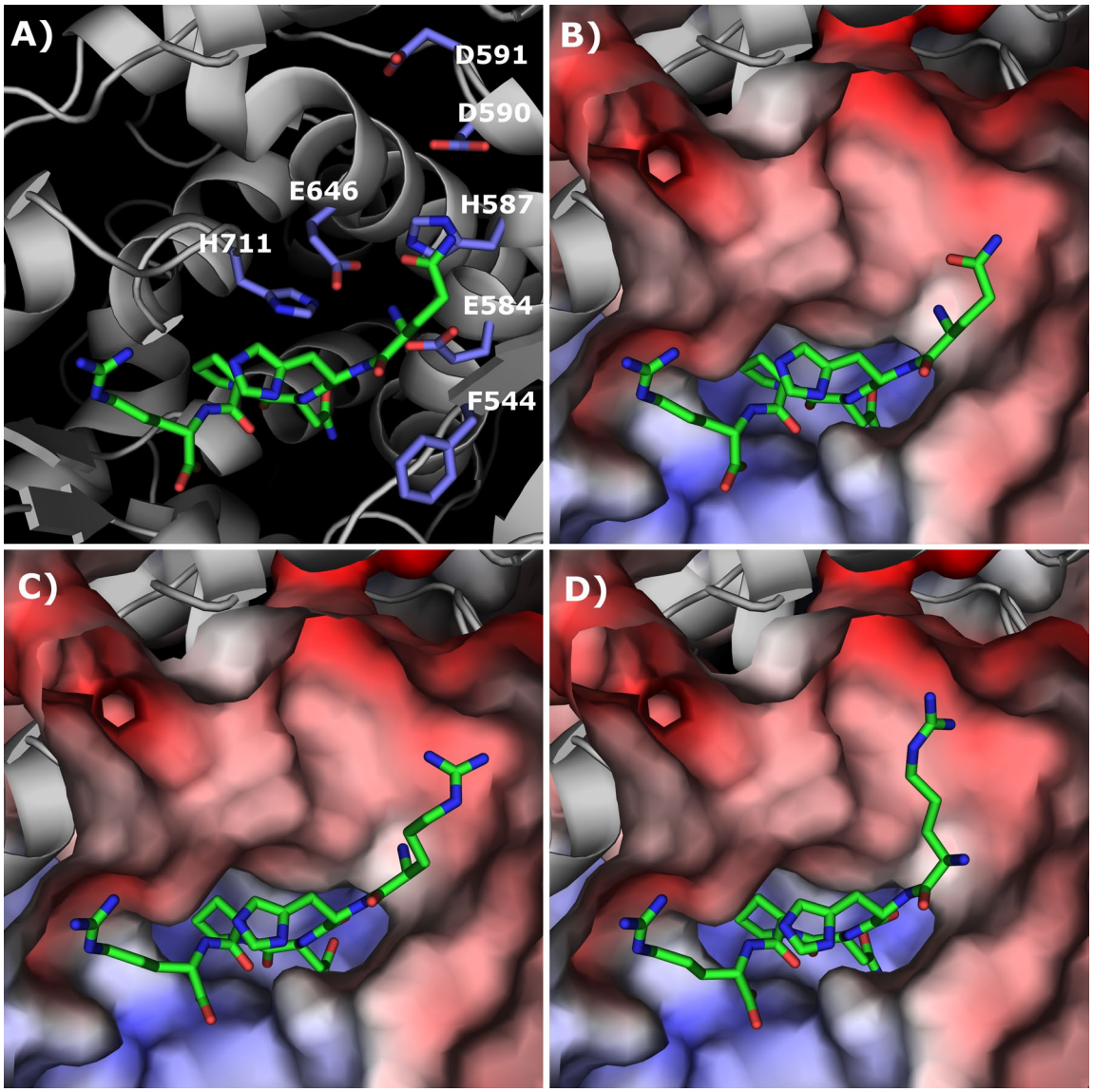

Fig. 1 **a**, **b** Theoretical complex of NEP–sialorphin, **c** model of NEP–peptide X, **d** model of NEP–peptide XI

I–XIII are presented in Table 2. The peptides modified in the first position with Ile^1 (peptide II), Val^1 (peptide IV), Lys^1 (peptide VI), and Leu^1 (peptide VIII) displayed a higher inhibitory potency for NEP as compared to that of sialorphin. Peptides X and XII containing in the first position, respectively, Arg and Orn, displayed equivalent inhibitory potency for NEP as compared to that of parent compound. Because plasma peptidases usually cleave L-amino acid peptide bonds, we decided to find out if using the D isomer of an amino acid would result in an enhanced inhibitory potency of a peptide. In this study, the change of the *N*-terminal amino acid configuration from L to D in peptides II, IV, VI, VIII and XII led to the abolition of their inhibitory activity (peptides III, V, VII, IX and XIII). Only with peptide X, changing the arginine configuration from L to D did not affect the inhibitory activity (peptide XI). Peptides X and XI exhibited comparable activities that were only slightly higher than that of sialorphin.

Stability in human plasma

Sialorphin, peptides II and XI were incubated in human plasma to determine their stability. As shown in Fig. 2, both displayed a greater stability in plasma than did sialorphin. After 2 h of incubation sialorphin was almost completely decomposed. Peptide II was also sensitive to degradation. The most stable was peptide XI. After 2 h of incubation the levels of peptides II and XI were, respectively, 4.7 and 17.9 times higher in relation to that

Table 1 Physicochemical properties of peptides I–XIII

Peptide	Name	Molecular formula	HPLC t_R [min]	$[M]^+$ calc.	$[M+H]^+$ found
I	Sialorphin (Gln-His-Asn-Pro-Arg)	$C_{26}H_{42}N_{12}O_8$	5.5**	650.3	651.4
II	[Ile^1]sialorphin	$C_{27}H_{45}N_{11}O_7$	7.09*	635.3	636.4
III	[D-Ile^1]sialorphin	$C_{27}H_{45}N_{11}O_7$	5.49*	635.3	636.4
IV	[Val^1]sialorphin	$C_{26}H_{43}N_{11}O_7$	4.82**	621.3	622.3
V	[D-Val^1]sialorphin	$C_{26}H_{43}N_{11}O_7$	5.85**	621.3	622.4
VI	[Lys^1]sialorphin	$C_{27}H_{46}N_{12}O_7$	5.59**	650.3	651.1
VII	[D-Lys^1]sialorphin	$C_{27}H_{46}N_{12}O_7$	5.05**	650.3	651.1
VIII	[Leu^1]sialorphin	$C_{27}H_{45}N_{11}O_7$	8.57*	635.3	636.5
IX	[D-Leu^1]sialorphin	$C_{27}H_{45}N_{11}O_7$	6.27**	635.3	636.2
X	[Arg^1]sialorphin	$C_{27}H_{46}N_{14}O_7$	6.26**	678.3	679.3
XI	[D-Arg^1]sialorphin	$C_{27}H_{46}N_{14}O_7$	5.72**	678.3	679.2
XII	[Orn^1]sialorphin	$C_{26}H_{44}N_{12}O_7$	5.17**	636.3	637.1
XIII	[D-Orn^1]sialorphin	$C_{26}H_{44}N_{12}O_7$	5.06**	636.3	637.5

*A linear gradient from 2–60% B in 15 min

**A linear gradient from 2 to 40% B in 15 min, where [A] 0,1% TFA in water, [B] 0.1% TFA in acetonitrile, column Kromasil C8 (4.6×250 mm, pore size 100 Å, particle size 5 µm), flow rate 1.5 mL/min, $\lambda = 214$ nm

Table 2 Degradation rates (k) and half-lives ($t_{1/2}$) of Met-enkephalin incubated with NEP alone and with peptides I–XIII

Peptide	Inhibitor	$1000 \times k$ [1/min]	$t_{1/2}$ [min]
	Without inhibitor	25.35 ± 1.05	27 ± 1
I	Sialorphin (Gln-His-Asn-Pro-Arg)	8.84 ± 0.27***	78 ± 2***
II	[Ile^1]sialorphin	5.32 ± 0.17***, ###	130 ± 4***, ###
III	[D-Ile^1]sialorphin	26.75 ± 0.60 ###	26 ± 1 ###
IV	[Val^1]sialorphin	6.20 ± 0.26***, ##	111 ± 4***, ##
V	[D-Val^1]sialorphin	25.22 ± 1.24 ###	27 ± 1 ###
VI	[Lys^1]sialorphin	6.51 ± 0.17***, ##	106 ± 3***, ##
VII	[D-Lys^1]sialorphin	21.14 ± 0.76*, ###	33 ± 1*, ###
VIII	[Leu^1]sialorphin	6.70 ± 0.10***, ##	103 ± 1***, ###
IX	[D-Leu^1]sialorphin	25.71 ± 0.59###	27 ± 1###
X	[Arg^1]sialorphin	8.34 ± 0.12***	83 ± 1***
XI	[D-Arg^1]sialorphin	7.17 ± 0.26***, #	96 ± 3***, ##
XII	[Orn^1]sialorphin	9.60 ± 0.39***	72 ± 3***
XIII	[D-Orn^1]sialorphin	20.88 ± 0.07*, ###	33 ± 1**, ###

Data are mean ± SEM

**$p < 0.01$, ***$p < 0.001$, compared to "without inhibitor", ##$p < 0.01$, ###$p < 0.001$, compared to peptide I

of sialorphin (I). Peptides I and II after 24 h of incubation in human plasma were completely decomposed, while as many as 80% of peptide XI was detected. After 48 h the level of peptide XI dropped to 45%. We showed that incorporation of the arginine residue in configuration D in the sialorphin sequence at position 1 would be effective in the protection of the peptide against plasma enzymatic degradation.

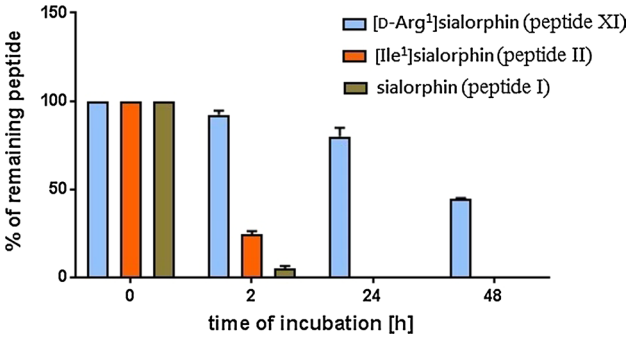

Fig. 2 Sialorphin and its analogues (peptides II and XI) stabilities in human plasma. The averages of the % of remaining peptide with a standard deviation are presented

Molecular modelling

Figure 1 shows visualization of the protein–peptide complexes. The protein shape is marked in grey (A), electrostatic surface (B,C,D) is coloured red (negative charge) and blue (positive charge). The ligand is marked green. Residues located on the protein–peptide interface are marked blue. Theoretical complex of the human NEP with sialorphin (based on 2QPJ structure) is shown in Fig. 1a, b. Model of NEP–peptide X ([Arg^1]sialorphin) and model of NEP–peptide XI ([D-Arg^1]sialorphin) are shown, respectively, in Fig. 1c, d. As we pointed out previously, the *N*-terminally blocked peptides by the acetyl group do not have inhibitory activity (Kamysz et al. 2016). According to our model the distance between *N*-terminus of the peptide and Glu^{584} and Glu^{646} is 3.2 and 3.1 Å, respectively (as measured between closest nitrogen and

oxygen atoms). With such close distance we may suspect a salt bridge interaction, which seems to be crucial for a good binding affinity of the peptide. The second evidence for the described statement is the negative charge of the protein binding grove (Fig. 1). Almost all (except peptide XI) residues in position 1 in D configuration are devoid of inhibitory activity. The explanation of this finding is rather simple and based on the described statement. The protein binding grove is rather thin, hence the inhibitor has to assume appropriate conformation to fit it (Fig. 1). With the L residues, it is possible to maintain interactions of the *N*-terminus with pointed glutamic acids and adapt a conformation of the side chain to fit the binding grove. This situation is unlikely with the D residues, since they are unable to create the pointed interactions. As a consequence, they do not show inhibitory activity. The only exception is D-Arg^{1}. In this case, the interactions of its *N*-terminus with Glu^{584} and Glu^{646} is replaced by a salt bridge formed between residue D-Arg^{1} of peptide XI and Asp^{590}. Another residue potentially capable to create the same set of interactions is D-Lys^{1} in peptide VII. However, this residue seems to be too short to create a strong salt bridge with Asp^{590}.

Conclusions

Proteolytic degradation of peptide bonds in sialorphin as well as in other peptides is considered as the main limitation of their use in systemic therapeutic applications. In addition, in sialorphin there is a problem with stability of the *N*-terminal glutamine residue. This amino acid can be converted into a cyclic structure called pyroglutamine (Glp) in the body fluids at pH < 7. Therefore, attempts were made to stabilize the sequences. In this study, we demonstrated that substitution of the Gln^{1} residue in sialorphin molecule with hydrophobic amino acid residues such as Ile, Leu, Val or amino acid residues with a positive side chain (Lys, Arg, Orn) leads to an increase in the NEP inhibitory activity. On the other hand, the change of amino acid configuration from L to D in position 1 in sialorphin analogues leads to abolition of the NEP inhibitory activity. However, introducing the D-Arg residue in position 1 to the sialorphin molecule (peptide XI) was a proper way to obtain a compound not only with increased inhibitory activity against NEP but also showing a significantly higher plasma stability than that of the parent molecule. Peptide XI exhibited inhibitory activity against NEP because only in this case the salt bridge could be created between the side chain of residue D-Arg^{1} of the peptide and residue Asp^{590} of the enzyme. Our results provide a useful information for designing NEP inhibitors with enhanced stability.

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Author contributions The manuscript was written through contributions of all authors and they approved its final version.

Compliance with ethical standards

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

Research involving human participants and/or animals This article does not contain studies with human or animal subjects performed by any of the authors that should be approved by Ethics Committee.

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