
# Appendix 3b

## SELF-PRESENTATION

## A STRUCTURE-ACTIVITY RELATIONSHIP STUDY OF BIOLOGICALLY ACTIVE PEPTIDES AND THEIR EFFECT ON MODEL MEMBRANES

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**2.** Diplomas and scientific degrees

**Ph.D.**
University of Gdansk, Faculty of Chemistry, Gdansk 2005.
Title: „Conformational investigations of vasopressin and its analogues using nuclear magnetic resonance spectroscopy."
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**Ch.E.**
Gdansk University of Technology, Faculty of Chemistry, Gdansk 2001.
Title: „Substancje biologicznie czynne pochodzenia roślinnego w kosmetykach."
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**M.Sc.**
University of Gdansk, Faculty of Chemistry and Institute of Meteorology and Water Economy, Gdansk 2000.
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**3.** Scientific employment

| | |
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| 11.04.2009-28.08.2009 | maternity leave |
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**4.** Indication of the achievement resulting from article 16 paragraph 2 of the act of 14 March 2003 on Academic Degrees and Titles and Degrees and Titles in Art (Journal of Laws No. 65, item 595, with subsequent amendments)

**A. Title of scientific achievement**

**A STRUCTURE-ACTIVITY RELATIONSHIP STUDY OF BIOLOGICALLY ACTIVE PEPTIDES AND THEIR EFFECT ON MODEL MEMBRANES**

**B. Publications included in the scientific achievement**

**H1. Sikorska E**✉, Kwiatkowska A, Sobolewski D, Ślusarz R, Ślusarz MJ. Influence of bulky 3,3-diphenylalanine enantiomers replacing position 2 of AVP analogues on their conformations: NMR and molecular modeling studies. *Eur. J. Med. Chem.* 2010, 45: 4065-4073. ($IF_{2010}$: 3,193)

**H2.** Lubecka E, Kwiatkowska A, Ciarkowski J, **Sikorska E**✉. NMR studies of new arginine vasopressin analogs modified with alpha-2-indanylglycine enantiomers at position 2 bound to sodium dodecyl sulfate micelles. *Biophys. Chem.* 2010, 151: 139-48. ($IF_{2010}$: 2,108)

**H3. Sikorska E**✉, Sobolewski D, Kwiatkowska A. Conformational preferences of proline derivatives incorporated into vasopressin analogues: NMR and molecular modelling studies. *Chem. Biol. Drug Des.* 2012, 79: 535-547. ($IF_{2012}$: 2,469)

**H4. Sikorska E**✉, Iłowska E, Wyrzykowski D, Kwiatkowska A. Membrane structure and interactions of peptide hormones with model lipid bilayers. *Biochem. Biophys. Acta* (*BBA)-Biomembranes* 2012, 1818: 2982-2993. ($IF_{2012}$: 3,389)

**H5. Sikorska E**✉, Kwiatkowska A. Micelle-bound conformations of neurohypophyseal hormone analogues modified with a Cα-disubstituted residue: NMR and molecular modeling studies. *J. Biomol. Struct. Dyn.* 2013, 31: 748-764. ($IF_{2013}$: 2,983)

**H6. Sikorska E**✉, Greber K, Rodziewicz-Motowidło S, Szultka Ł, Łukasiak J, Kamysz W. Synthesis and antimicrobial activity of truncated fragments and analogs of citropin 1.1: The solution structure of the SDS micelle-bound citropin-like peptides. *J. Struct. Biol.* 2009, 168: 250-258. ($IF_{2009}$: 3,673)

**H7. Sikorska E**✉, Dawgul M, Greber K, Iłowska E, Pogorzelska A, Kamysz W. Self-assembly and interactions of short antimicrobial cationic lipopeptides with membrane lipids: ITC, FTIR and molecular dynamics studies. *Biochem. Biophys. Acta* (*BBA)-Biomembranes* 2014, 1838: 2625-2634. ($IF_{2014}$: 3,836)

**H8. Sikorska E**✉, Kamysz E. Effect of head-to-tail cyclization on conformation of histatin-5. *J. Pept. Sci.* 2014, 20: 952-957. ($IF_{2014}$: 1,546)

**H9. Sikorska E**✉, Wyrzykowski D, Szutkowski K, Greber K, Lubecka EA, Zhukov I. Thermodynamics, size and dynamics of zwitterionic dodecylphosphocholine and anionic sodium dodecyl sulfate mixed micelles. *J. Therm. Anal. Calorim.* 2015, DOI: 10.1007/s10973-015-4918-0. ($IF_{2014}$: 2,042)

Total IF for publications included in the scientific achievement is **25.239** (calculated on the basis of IF values from the year of publication). Average IF per publication is 2.8.

## C. Discussion on the publications being a scientific achievement

## INDRODUCTION

**The subjects of research reported in the scientific achievement encompassed two groups of peptides: neurohypohyseal homones (NPHs) and antimicrobial peptides (AMPs).** Despite the fact that the subjects are highly diverse in terms of biological functions they perform, their common feature is the ability to interact with the biological membranes. In the case of NPHs, the interactions with membrane are only an intermediate step in the way to the appropriate GPCR receptor.[1-3] In turn, in the case of AMPs, the interactions with lipid membrane result usually in disintegration of the membrane by forming channels in it, its depolarization or fragmentation, which leads to cell death[4,5] (Figure 1).

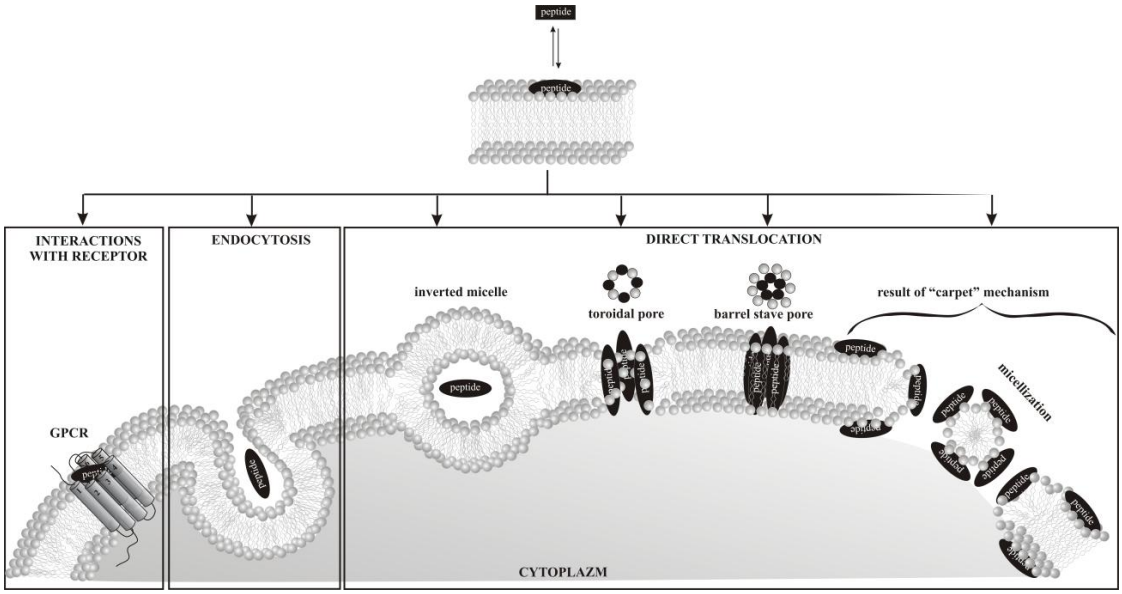


**FIGURE 1**
Peptide–membrane interaction patterns in relation to peptide function (modified from Trabulo et al.[6]).

Bearing in mind that the lipid bilayer plays a fundamental role in the activity of both groups of peptides, it was important to determine their conformations under conditions similar to those prevailing in the environment of the biological membrane. Solvents such as DMSO (dimethyl sulphoxide) or TFE (trifluoroethanol) are used in NMR spectroscopy as membrane-mimicking ones.[7-9] However, the DPC (dodecylphosphocholine) and SDS (sodium dodecyl sulphate) micelles are believed to be much better models of biological membranes, compatible with NMR spectroscopy.[10,11] The use of SDS to imitate membrane environment is not always rational, because it has no structural analogues among the phospholipids that are components of biological membranes. Nevertheless, due to negative surface charge, SDS micelles are commonly used to mimic bacterial membranes. In turn, DPC provides a zwitterionic surface on the micelle that adequately mimics eukaryotic membranes.[12] However, the surface of most biomembranes is negatively charged. Typically,

10-20% of the membrane lipids are anionic.[13] Thus, to mimic electrostatic properties of the eukaryotic membranes, a membrane model characterised by a slight prevalence of the negative charge, such as that in the DPC/SDS mixed micelles, can be used as well.[14-16] Both SDS and DPC micelles have extensively been studied and reported in the literature. There are also known their three-dimensional models used in molecular dynamics simulations including peptide-micelle interactions[10,17-27] In turn, for the mixed DPC/SDS micelles, only the results of studies performed by Scorciapino and co-workers have been reported in the literature.[28] **Therefore, I performed studies on the mixed DPC/SDS micelles to determine an influence of SDS on critical micelle concentration (*cmc*), thermodynamics of micellization processes, as well as the size and dynamics of the mixed micelles**. In this part of my studies, I used the same methods as those for conformational analysis of the peptides and peptide-lipid interactions.

The papers displayed in self-presentation are divided into three parts: "Neurohyphophyseal hormones" [**H1-H5**], „Antimicrobial peptides" [**H6-H8**] and "A characteristic of the mixed DPC/SDS micelles used in the conformational studies of peptides as biological membrane models" [**H9**].

## NEUROHYPOPHYSEAL HORMONES

Neurohypophyseal hormones are cyclic nonapeptides with a 20-membered tocin ring closed by a disulfide bridge between $Cys^1$ and $Cys^6$ and a C-terminal linear tripeptidic amidated tail. They are synthesised in both the supraoptic and the paraventricular nuclei of the hypothalamus as a fragment of a larger precursor protein, the so-called preprohormone. Enzymatic cleavage of the preprohormone occurs in secretory granules transported along the long axons to the posterior pituitary, where the components of the preprohormone are stored. The contents of the granules appear to be released by exocytosis when the nerve endings are depolarised.[29-31]

Arginine vasopressin (AVP, CYFQNCPRG-$NH_2$) and oxytocin (OT, CYIQNCPLG-$NH_2$) are naturally occurring neurohypophyseal hormones in most mammals. They are of particular interest because of their important pharmacological properties. Vasopressin controls urine concentration and the blood pressure.[32] Moreover, it is responsible for stimulation of the adrenocorticotropine (ACTH) secretion from the anterior pituitary.[33] These different biological activities of AVP are mediated by three receptor subtypes: $V_2$ (renal), $V_{1a}$ (vasopressor) and $V_{1b}$ (pituitary), being typical members of class A GPCR, which are membrane-spanning proteins.[34-36] Besides, vasopressin participates in stability of the body temperature[37] and in the stimulation of platelet aggregation.[38] It is also secreted under stress conditions.[39] In turn, the oxytocin functions are mainly related to the reproductive system. The primary physiological function of OT is to induce contractions of uterine smooth muscle and mammary myoepithelium. Besides, OT initiates the maternal behaviour. All effects induced by oxytocin are the result of binding to one type of GPCR receptor.[34]

A significant sequence similarity of AVP and OT causes that they interact with both vasopressin and oxytocin receptors. However, a weaker effectiveness has been reported for cross-interactions.[40]

A lipid bilayer is an important mediator in the peptide-receptor binding. The current model of peptide interactions with GPCRs suggests that the bioactive conformation of the former is induced upon association with the cell membrane followed by a two dimensional diffusion process, whereby the ligand is recognised and then interacts with the receptor.[1,2,9,41] The lipid bilayer has also a massive impact on the activity and function of GPCR receptors.[42-50] According to multiple active GPCR states, GPCR receptors exist in many states of activation in the absence of a ligand (multiple active GPCR states).[51] For simplicity, a two-states model defining only two states of the receptor, the active (R*) and inactive (R) has often been employed. Only the receptors in the R* state can couple to, and activate G-proteins. Agonists both stabilize and increase the fraction of the active state. Interactions of the so-called "neutral antagonist" with receptor do not alter the equilibrium between the inactive and active states, because they exhibit the same affinity to both states of the receptor. However, some ligands with antagonistic properties stabilize and enrich inactive state of receptor shifting the equilibrium to the latter.[52,53] These are the so-called "inverse agonists". It should be emphasised that usually the "neutral antagonists" and the "inverse agonists" are not distinguished in the bioassay of neurohypophyseal analogues and both groups are referred to as antagonists.

Bearing all of this in mind, it might be hypothesised that indirectly, through altering membrane physical properties in which the receptor is embedded, a ligand may modify the function of receptor and shift equilibrium either to the active or inactive state. Consequently, it may affect indirectly effectiveness of agonist or antagonist binding.

Statistical data show that preterm birth, affecting about 10% of all births, is the major cause of perinatal mortality and morbidity and has remained unchanged over the last four decades despite intensive antenatal care programmes aimed at high-risk group, the widespread use of tocolytics, and a series of other preventive and therapeutic interventions.[54-58] In the context of these data, the antagonists of OT receptors are ones of the most promising solutions to the problem of preterm births. Therefore, the studies on neurohypophyseal hormone analogues were focused first of all on antagonists of oxytocin receptors (Table 1). Five of the peptides were selective OT antagonists and three were more potent OT antagonist than atosiban ([Mpa$^{1}$,D-Tyr(Et)$^{2}$,Thr$^{4}$,Orn$^{8}$]OT) – the currently available tocolytic agent registered under the trade name TRACTOCILE. However, similar to atosiban,[59] they were non-selective.

**TABLE 1**

Neurohypophyseal hormone analogues selected for conformational studies and their activities.[40,60-66]

| Peptide | Activity | | | No. of paper in scientific achievement |
|---|---|---|---|---|
| | OT | $V_{1a}$ | $V_2$ | |
| **[Mpa$^1$,Dpa$^2$,Val$^4$,D-Arg$^8$]VP** | **very weak antagonist** | inactive | **agonist** | **H1** |
| **[Mpa$^1$,D-Dpa$^2$,Val$^4$,D-Arg$^8$]VP** | **antagonist** | **weak antagonist** | **strong agonist** | |
| **[D-Dpa$^2$,D-Arg$^8$]VP** | **antagonist** | inactive | **strong agonist** | |
| **[Mpa$^1$,D-Dpa$^2$]AVP** | **antagonist** | inactive | **strong agonist** | |
| **[Igl$^2$]AVP** | **antagonist** | inactive | negligible | **H2** |
| **[Mpa$^1$,Igl$^2$]AVP** | **antagonist** | inactive | negligible | |
| **[D-Igl$^2$]AVP** | **strong antagonist** | **weak antagonist** | negligible | |
| **[Mpa$^1$,D-Igl$^2$]AVP** | **strong antagonist** | **weak antagonist** | negligible | |
| **[Nmp$^2$]AVP** | **antagonist** | **weak antagonist** | negligible | **H3** |
| **[Nmp$^2$,D-Arg$^8$]AVP** | **antagonist** | **weak antagonist** | inactive | |
| **[APy$^{2\text{-}3}$]AVP** | inactive | inactive | inactive | |
| **[Apy$^{2\text{-}3}$]AVP** | inactive | inactive | inactive | |
| **[Mpa$^1$,Ica$^2$,D-Arg$^8$]VP** | **antagonist** | inactive | negligible | |
| **OT (CYIQNCPLG-$NH_2$)** | **agonist** | **very weak agonist** | **very weak agonist** | **H4** |
| **AVP (CYFQNCPRG-$NH_2$)** | **very weak agonist** | **agonist** | **agonist** | |
| **[D-Igl$^2$]AVP** | **strong antagonist** | **antagonist** | negligible | |
| **[Adg$^2$,D-Arg$^8$]VP** | **very weak agonist** | inactive | negligible | |
| **[*cis*-Apc$^2$]OT** | **antagonist** | **weak antagonist** | nd | |
| **t-Bba[*cis*-Apc$^2$,Val$^4$]AVP** | **very weak agonist** | inactive | inactive | **H5** |
| **Aba[*cis*-Apc$^2$,Val$^4$]AVP** | **antagonist** | inactive | inactive | |
| **Aca[*cis*-Apc$^2$,Val$^4$]AVP** | **weak antagonist** | inactive | inactive | |
| **[*cis*-Apc$^2$]OT** | **antagonist** | **weak antagonist** | nd | |
| **[Mpa$^1$,*cis*-Apc$^2$]OT** | **strong antagonist** | **weak antagonist** | nd | |

Aba, 4-aminobenzoic acid; Aca, 1-adamantane carboxylic acid; Adg, (S)-2-(1-adamantyl)glycine; Apy, (2R,4S)-4-aminopyroglutamic acid; APy, (2S,4S)-4-aminopyroglutamic acid; Ica, indoline-2-carboxylic acid; cis-Apc, *cis*-1-amino-4-phenylcyclohexane-1-carboxylic acid; Dpa, 3,3-diphenylalanine; Igl, α-2-indanylglycine; Mpa, 3-mercaptopropionic acid; Nmp, (2S,4R)-4-(naphthalene-2-ylmethyl)pyrrolidine-2-carboxylic acid; t-Bba, 4-*tert*-butylbenzoic acid.
nd - no data available

Most of the peptides studied are sparingly water-soluble, this being a serious challenge for the preparation of samples with concentrations necessary for NMR studies. Due to their poor solubility, the NMR spectra of the first studied analogues, those modified with 3,3-diphenlalanine [**H1**], were recorded in a DMSO-$d_6$ solution. NMR measurements of analogues modified with α-2-indanylglycine were performed in SDS micelles [**H2**]. The SDS micelles solubilized the peptides and enhanced their water solubility. The conformational studies of the remaining analogues were carried out in the DPC/SDS mixed micelles. However, due to the solubility problem of the peptides, they were first dissolved in perdeuterated methanol-$d_3$ and then the samples were diluted with a aqueous solution of the DPC/SDS mixed micelles [**H3** and **H5**].

To find the interactions between the peptides and the hydrophobic core of the mixed micelles, n-doxyl stearic acid derivative (16-doxyl-stearic acid, 16-DSA) was used. The 16-doxylstearic acid (16-DSA) incorporates into the micelles with the free radical group positioned close to the centre of the micelle. Consequently, it induces a broadening, as a result of shortening the $T_2$ relaxation time, of signals from peptide residues that are close to the micelle centre.[67,68] Interpretation of signal intensity reduction upon addition of the 16-DSA allowed to determine location of the peptide within the micelle (Figure 2).

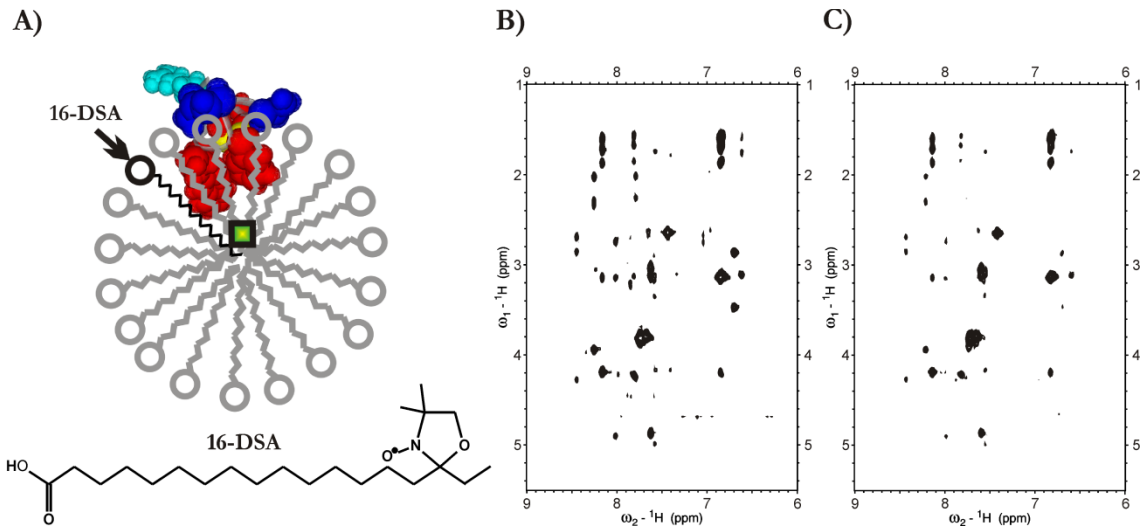


**FIGURE 2**

(**A**) The use of 16-doxyl-stearic acid (16-DSA) to identify interactions between [Nmp$^2$,D-Arg$^8$]VP and micelle [**H3**]. (**B**) The TOCSY spectra of [Nmp$^2$,D-Arg$^8$]VP recorded in the 5:1 DPC/SDS mixed micelles without 16-DSA and (**C**) with 16-DSA added.

The analogues studied revealed a tendency to create β-turns at positions 2,3 and/or 3,4, which indicates that this structural element is characteristic of ligands of OT and $V_{1a}$ receptors. The β-turn in fragment 1-4 seems to be crucial for antipressor activity, but it is not necessary for antiuterotonic one [**H1-H3**, **H5**]. Among the peptides with antidiuretic activity, the β-turn in the Cys$^6$-Gly$^9$ fragment seems to enhance the antidiuretic activity, though not being crucial for its appearance [**H1**].

Results of previous studies on peptide-receptor interactions suggest that the interactions between aromatic residues of the ligand and aromatic residues located on TM6 of $V_{1a}$ and OT receptors are characteristic of antagonists binding.[69-71] It is believed that these interactions prevent conformational changes of TM6 helix of the receptors and initiate reverse to their inactive state.[69,70]

A detailed examination of the peptide-micelle interactions have indicated that the aromatic side chains are immersed into the hydrophobic core of the micelles, whereas a positively charged Arg$^8$ side chain either interacts with negatively charged fragments of the micelle or is exposed to the aqueous phase. An exception provides the [Mpa$^1$,Ica$^2$,D-Arg$^8$]VP analogue [**H3**], in which the Ica$^2$ side chain lies on a different side of the tocin ring plane than does Phe$^3$. This mutual arrangement of the Ica$^2$ and Phe$^3$ side chains precludes

immersion of both into the micelle core. However, the surface accessible of $Ica^2$ is noticeably reduced to avoid unfavourable interactions between its hydrophobic side chain and the polar aqueous environment. A characteristic feature of this analogue is also *cis* $Mpa^1$-$Ica^2$ peptide bond.

An interesting case provided analogues modified with 4-aminopyroglutamic acid, [$APy^{2-3}$]AVP and [$Apy^{2-3}$]AVP [**H3**]. They were completely inactive in all of the biological tests and at the same time they only weakly interacted with the micelle. These peptides did not penetrate into the hydrophobic core of the DPC/SDS mixed micelle and were only weakly associated with its surface. In the latter case, the TOCSY spectrum displayed more than two sets of signals for the $Gln^4$-$Arg^8$ fragment. Multiplying resonances in the NMR spectra are the result of a slow conformational exchange on the experimental time scale.[72] With [$Apy^{2-3}$]AVP, the presence of more than two sets of proton resonances may be the consequence of both *cis*/*trans* isomerization of $Cys^6$-$Pro^7$ peptide bond and/or an equilibrium set up between different micelle-bound states of the peptide.

Previous studies on interactions between neurohypophyseal hormone-like peptides with VP/OT receptors indicated that binding antagonists as well as antagonists' conformations in the binding pocket of the receptor may be either similar or completely different from those of the agonists.[73-75] Examination of analogues modified with the *cis*-Apc residue has shown no clear differences between the conformations adopted by the agonist and that of antagonist [**H5**]. Among the AVP-like peptides, only t-Bba[*cis*-$Apc^2$,$Val^4$]AVP is a weak agonist of OT receptors. It is surprising, because $Tyr^2$ naturally occurring in AVP and OT, and replaced in this analogue by *cis*-Apc, is believed to be responsible for receptor activation and signal transduction.[76,77] As expected, the remaining two AVP analogues, Aba[*cis*-$Apc^2$,$Val^4$]AVP and Aca[*cis*-$Apc^2$,$Val^4$]AVP are OTR antagonists. A comparison of the three-dimensional structures of Bba[*cis*-$Apc^2$,$Val^4$]AVP and Aca[*cis*-$Apc^2$,$Val^4$]AVP showed a close similarity in the arrangement of side chains, despite the different activities. On the other hand, inspection of the peptide-micelle interactions has shown that $Asn^5$ and $Gly^9$ of the former are more exposed to aqueous phase than those in the remaining analogues. It is known that $Asn^5$, similar to $Tyr^2$, is critical for signal transduction. In turn, $Gly^9$ is supposed to be important for selectivity against OT receptor. Besides, it is also believed that the terminal $CONH_2$ group may affect the binding efficiency.[76,78] Therefore, those differences in interactions between Bba[*cis*-$Apc^2$,$Val^4$]AVP and Aca[*cis*-$Apc^2$,$Val^4$]AVP and the micelle might be responsible for their different activity.

A closer inspection of interactions of the *cis*-Apc modified peptides with the micelle indicates that the arrangement of the AVP-peptides is nearly perpendicular, whereas that of the OT-like ones is rather parallel to the micelle surface. These results are compatible with polarity of the C-terminal part of the peptides. Position 8 of the AVP and OT analogues is occupied by a positively charged Arg and the nonpolar leucine, respectively. Consequently, in the case of the former, the C-terminus is exposed to the polar aqueous environment, whereas in the latter it is immersed into the hydrophobic core of the micelle. These findings are consistent with the studies on interactions responsible for AVP and OT

binding to human neurohypophyseal hormone receptors, where different locations of both peptides in the receptor cavity have been reported.[78,79]

The studies on interactions with liposomes were conducted for selected peptides only, namely for AVP, OT, [D-Igl$^2$]AVP, [Adg$^2$,D-Arg$^8$]VP and [*cis*-Apc$^2$]OT [**H4**]. Conformational changes in the peptides upon binding to liposomes were examined using CD spectra at different temperatures. The influence of the peptides on the lipid acyl chain order and the temperature of phase transition of the lipids were determined using FTIR spectroscopy. In addition, to determine the binding parameters of the peptides to lipids, isothermal titration calorimetry (ITC) was employed. ITC measurements usually enable determination of the binding constants, reaction stoichiometry, enthalpy, and entropy. However, practical range of measurable binding constants by ITC is restricted to values of $10^3$-$10^8$ $M^{-1}$.[80] With the neurohypophyseal hormones, the interactions with lipids are probably too weak to be measured by ITC.

Interpretation of the FTIR spectra taken at different temperatures has shown that the peptides affect main transition points of the lipids through lowering them by 1.2-1.6°C. The [Adg$^2$,D-Arg$^8$]VP analogue, with a negligible activity, suppressed the membrane fluidity in the liquid crystalline phase. The changes of the membrane structure around the receptor induced by the peptide may negatively affect the ligand-binding parameters of the receptor.[46-49] This seems reasonable, because Gurdal et al.[44] found a correlation between the membrane fluidity and impaired coupling of the G protein with the $\beta_2$-adrenergic receptor.

A closer examination of the CD spectra indicated that the Tyr$^2$ side chain is more exposed outside the molecule in liposome-bound oxytocin than in the water. In turn, as the result of AVP-lipid binding, the stacking interactions between aromatic tyrosine and phenylalanine are diminished, which is probably due to immersion of both aromatic rings in hydrophobic core of the liposome. The bulky and hydrophobic residues incorporated into the N-terminal part of neurohypophyseal hormones enhance the hydrophobic interactions between peptide and the lipid bilayer. Above the phase transition point of the lipids, the choline groups of the liposome are folded inward towards the surface, exposing more the negatively charged phosphatidyl groups.[81] Thus, the possibility of electrostatic interactions between the positively charged Arg$^8$ and negatively charged phosphatidyl groups is rising, which may affect the conformation of the C-terminal part of the AVP-like peptides.

## ANTIMICROBIAL PEPTIDES

The rapid growth of bacterial resistance to conventional antibiotics puts an increased challenge to the search of alternative treatment strategies. Antimicrobial peptides (AMPs) constitute part of the host innate immunity of living organisms of all types and are one of the most promising classes of compounds exhibiting antimicrobial activities. These peptides are rapidly mobilised to neutralize a wide range of microbes, including bacteria, fungi, viruses and even protozoa.[82,83] Based on their specific structure one can design analogues with enhanced activities and improved pharmacokinetic properties. For this

reason, they have attracted much attention owing to their application as therapeutic agents and preservatives.[84,85]

The following antimicrobial peptides were studied within the framework of scientific achievement: citropin 1.1 and its two truncated analogues, (1-12)citropin and [Ala$^4$](1-13)citropin, the head-to-tail cyclic analogue of histatin 5, and three synthetic lipopeptides, $C_{16}$-KK-$NH_2$, $C_{16}$-KGK-$NH_2$ and $C_{16}$-KKKK-$NH_2$.

## CITROPIN 1.1 AND ITS ANALOGUES

Citropin 1.1 is a 16-amino acid peptide (GLFDVIKKVASVIGGL-$NH_2$) isolated from the skin of the Australian green tree frog *Litoria citropa*.[86,87] It exhibits multifaceted biological activities, including widespectrum antimicrobial and anticancer activity, together with inhibition of nitric oxide synthase.[87-89] The N-terminal Gly-Leu-Phe fragment, as well as cationic residues located at positions 7 and 8, are essential for maximal expression of citropin 1.1 activity.[87] The studies performed in the framework of scientific achievement have shown that the progressive shortening of the C-terminal part suppresses the potency of the analogues [**H6**]. However, combining both the shortening of the C-terminal fragment and replacement of Asp$^4$ with Ala may result in an increase in potency against selected bacterial strains.

Previous conformational studies on citropina 1.1 in 50% TFE have shown the presence of α-helical structure extending along the entire peptide chain.[86] However, this structure is not retained in SDS micellar solution, where citropina 1.1 adopts two α-helices in fragments 4-7 and 10-16, separated by β-turn at position 8,9 [**H6**]. However, it cannot be excluded that the helix-break observed in citropin 1.1 is due to the spherical shape of SDS micelles. A characteristic conformational feature of citropina 1.1 in SDS micellar solution is also a salt bridge between the negatively charged side chain of Asp$^4$ and positively charged side chain of Lys$^7$. The (1-12)citropin assumes an α-helical structure in the Gly$^1$-Val$^9$ fragment terminated by βI-turn at position 9,10. This analogue exhibits the lowest antibacterial activities despite the presence of a regular α-helix over almost its entire length. In turn, (1-13)[Ala$^4$]citropin displays a higher activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) than citropin 1.1 and it demonstrates the tendency to adopt only a short α-helix in the middle part. Moreover, the conversion of α-helix to $3_{10}$-helix has been noticed in about 30% of conformations. The $3_{10}$-helical units could be thermodynamic intermediates during folding and unfolding of the α-helical segment of the peptide.[90] The common feature of all the conformations is a βI'-turn at position 10,11 stabilised by a HN$^{12}$-CO$^9$ hydrogen bond.

The negatively charged Asp$^4$ is strongly exposed to the aqueous phase in both citropin 1.1 and (1-12)citropin. This is the consequence of electrostatic repulsion between negatively charged Asp side chain and sulphate groups of SDS micelle. Replacement of Asp$^4$ with neutral Ala did not influence the arrangement of the side chain of the residue at position 4 in (1-13)[Ala$^4$]citropin compared to that of the parent peptide. In turn, shortening of the peptide backbone clearly resulted in a deeper immersion of both analogues in the hydrophobic core of the micelle compared to citropina 1.1[**H6**].

## THE HEAD-TO-TAIL CYCLIC HISTATIN 5

Histatins constitute a distinct family of at least 12 low-molecular-weight, histidine-rich, cationic, salivary peptides, of which histatins 1, 3, and 5 are the most abundant.[91] *In vitro* studies have demonstrated antifungal activity of histatins against a wide range of pathogenic fungi, including *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*[92-94] and against bacteria, *Streptococcus mutans* and *Porphyromonas gingivalis*.[95] Among the histatins, histatin 5 has been reported to be the most efficient in killing *Candida albicans,*[96] but it is also susceptible to proteolytic degradation.[97,98] One of the common strategies to increase peptide stability is cyclization. Moreover, cyclic peptides have often shown enhanced biological activity.[99-102] Histatin 5 and its head-to-tail cyclic analogue (c-Hst5) exhibited similar antimicrobial activities in all performed experiments.[103] The conformational studies using CD showed that the cyclic variant of histatin 5 occurred predominantly in helical conformation (~70%) in TFE solution. By analogy to histatin 5, the presence of zinc ions was required to induce helical conformation in the presence of negatively charged micelles.[103] The specific action of the $Zn^{2+}$ ions is attributed to the presence of a zinc-binding motif, His-Glu-X-X-His.[104]

Preliminary NMR spectra of the head-to-tail cyclic histatin 5 were recorded in the presence of SDS micelles. Unfortunately, the spectra were characterised by an extremely poor resolution due to the highly broadened signals. Therefore, the spectra of c-Hst5 were ultimately collected in a TFE solution [**H8**].

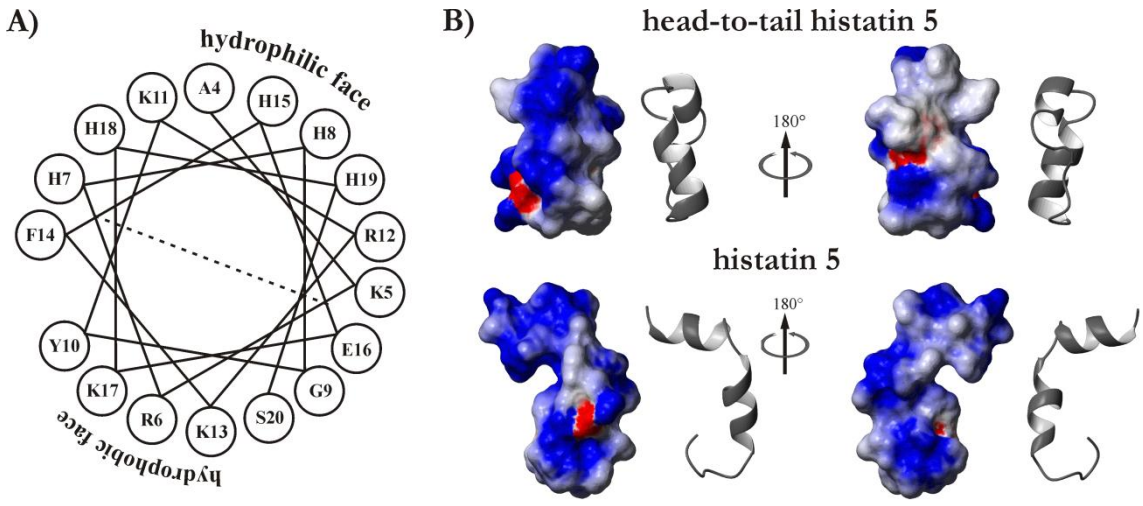


**FIGURE 3**

(**A**) Helical-wheel presentation of residues 4-20 of histatin 5 comprising both α-helical segments found in the head-to-tail cyclic analogue. (**B**) Surface electrostatic potential map of the head-to-tail cyclic analogue of histatin 5 and naturally occurring linear histatin 5. The figures were generated by the MOLMOL programme.[105]

The cyclic histatin 5 reveals a helix-loop-helix motif with α-helices at positions $Ala^4$-$His^7$ and $Lys^{11}$-$Ser^{20}$. Both helical segments are arranged relative to each other at an angle of *ca.* 142°. The helical-wheel presentation of the $Ala^4$-$Ser^{20}$ fragment (Figure 3) comprising both α-helical segments shows low amphipathic properties of histatin 5. However, taking into account the electrostatic potential surface of the entire molecule (Figure 3), it seems that one side of the peptide is more positively charged than the other. The negatively charged residues $Asp^1$ and $Glu^{16}$ are hidden in the interior of the peptide molecule. A comparison of the structures of the linear and cyclic histatin 5 indicates that the cyclization improves the polarised distribution of hydrophobic and hydrophilic residues (Figure 3).

## SYNTHETIC LIPOPEPTIDES

Short synthetic lipopeptides containing cationic amino acids and fragments of fatty acids seem to be an interesting alternative to conventional antibiotics. They satisfy the amphipathicity and total positive charge conditions, essential features of antibiotic peptides. The lipopeptides studied in scientific achievement [**H7**], in addition to the antibacterial activity exhibit surface active properties. Thus, they can act as preservatives and surfactants allowing to obtain and secure the multiphase system stability.

In this group of compounds, I focused primarily on research on micellization processes and interactions of the lipopeptides with models of biological membranes. The lipopeptides studied exhibit a comparable activity against selected strains of both Gram-positive and Gram-negative bacteria. The minimum inhibitory concentrations (MICs) of the lipopeptides ranged from 4 to 16 μg/mL, while the minimal bactericidal concentrations (MBCs) ranged from 4 to 32 μg/mL. Haemolytic activity studies against human erythrocyte cells revealed that $C_{16}$-KK-$NH_2$ showed the highest haemolytic effectiveness [**H7**]. The critical micellar concentrations (*cmc*s) obtained from surface tension measurements revealed that *cmc* increased in the following order: $C_{16}$-KK-$NH_2$ < $C_{16}$-KGK-$NH_2$ < $C_{16}$-KKKK-$NH_2$. In addition, molecular dynamics simulations of the micellization processes showed that the aggregation numbers decreased with increasing of *cmc* values. This is due to steric interactions within the polar part of the micelles formed.

Analysis of interactions between lipopeptides and models of biological membranes using infrared spectroscopy (FTIR) and isothermal titration calorimetry (ITC) brought quite surprising results. The FTIR spectra showed that the lipopeptides had a significant influence on the hydrophobic core region of the DPPC membrane. The palmitic acid tail penetrates into the hydrophobic core of liposome and facilitates anchoring of the peptide into the lipid bilayer. As expected, $C_{16}$-KK-$NH_2$ and $C_{16}$-KGK-$NH_2$ interact also with negatively charged liposomes (DPPG or POPG) imitating bacterial membrane. These results provide support for an important role played by electrostatic interactions in the binding of the peptides to the membrane surface. In turn, $C_{16}$-KKKK-$NH_2$ has the highest overall positive charge among the peptides studied. However, contrary to expectations, both FTIR and ITC results suggest no electrostatic interactions with negatively charged liposomes [**H7**]. Nevertheless, similar results have previously been obtained for a five-

residue peptide, $Lys_5$.[106] This is a consequence of the substantial compensation of electrostatic and hydrophobic interactions between the positively charged peptide and the negatively charged membrane.

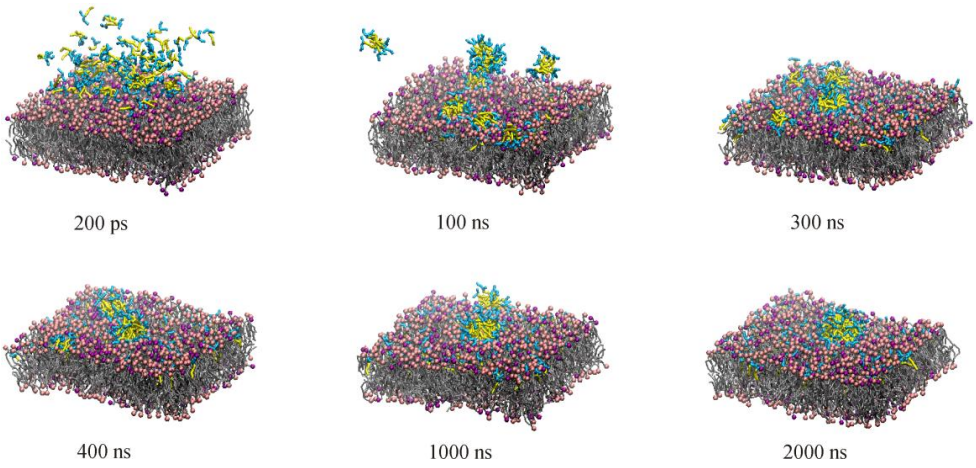


**FIGURE 4**

Snapshots from the 1:3 POPE:POPG binding simulations for $C_{16}$-KK-$NH_2$. Fatty acid tails are coloured yellow, while lysines are coloured cyan. Lipid tails are coloured gray, while lipid head groups are coloured pink and purple for POPG and POPE, respectively.

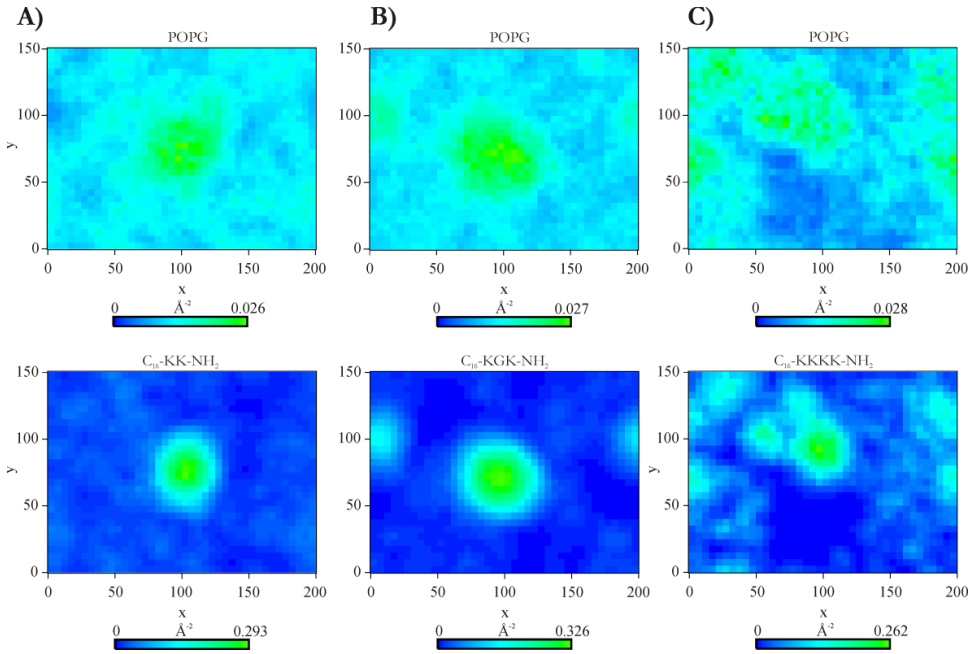


**FIGURE 5**

2D density map of the lipopeptides and POPG lipids in the upper leaflet of the membrane based on the last 100 ns of a total of 2 μs CG MD simulations. (**A**) $C_{16}$-KK-$NH_2$, (**B**) $C_{16}$-KGK-$NH_2$ and (**C**) $C_{16}$-KKKK-$NH_2$.

To visualise interactions between lipopeptides and bacterial membrane, I performed the coarse-grained molecular dynamics simulations using GROMACS 4.5.5 package.[107]

In subsequent steps of the CG MD simulations, three processes have been distinguished: formation of lipopeptide micelles associated with the surface membrane, insertion of the lipopeptide monomers into the lipid bilayer and insertion of lipopeptide micelles into the lipid bilayer (Figure 4).The lipopeptide micelles associated with membrane surface affect the membrane structure through a distinct enrichment of POPG lipids at the binding site (Figure 5).

## A CHARACTERISTIC OF THE MIXED DPC/SDS MICELLES USED IN CONFORMATIONAL STUDIES OF PEPTIDES AS BIOLOGICAL MEMBRANE MODELS

The structural studies of membrane-active peptides by solution NMR spectroscopy require membrane-mimicking environment for proper folding and stability.[2,6,83,108,109] Among the most prominent membrane models suitable for the peptide-membrane interaction studies, the similarity to biological membranes decreases in the order: liposomes, bicelles, mixed micelles and micelles.[110] The NMR studies on peptides in the presence of liposomes are typically limited to the solid state due to their considerable size. In solution NMR, the slow molecular tumbling resulting from the liposome size leads to a broadening of the NMR resonances, which makes achievement of a high-resolution 3D structure of the liposome-bound peptide difficult or even impossible.[111,112] In turn, micelles stand for a convenient compromise between a suitable membrane model and the need for relatively fast tumbling system in view of their short rotational correlation time.[113]

In scientific achievement, I studied the DPC/SDS mixed micelles, because they were subsequently used as a membrane model in conformational analysis of neurohypophyseal hormone analogues. The isothermal titration calorimetry (ITC) was used to determine the critical micelle concentrations (*cmc*s) and thermodynamic parameters of micellization processes. The dynamics and sizes of the micelles were deduced from the NMR spectra [**H9**].

The results indicate that the DPC/SDS mixed systems show the tendency to form two kinds of micelles in phosphate buffer solution (PBS). An increase in temperature or transfer of the mixed micelles from the buffered to unbuffered solution result in only a single micellization process, which reveals the formation of only one kind of the micelle (Figure 6). Addition of SDS to the DPC micelle results in a decrease in the *cmc* values. This indicative of the synergism in formation of the mixed micelles.

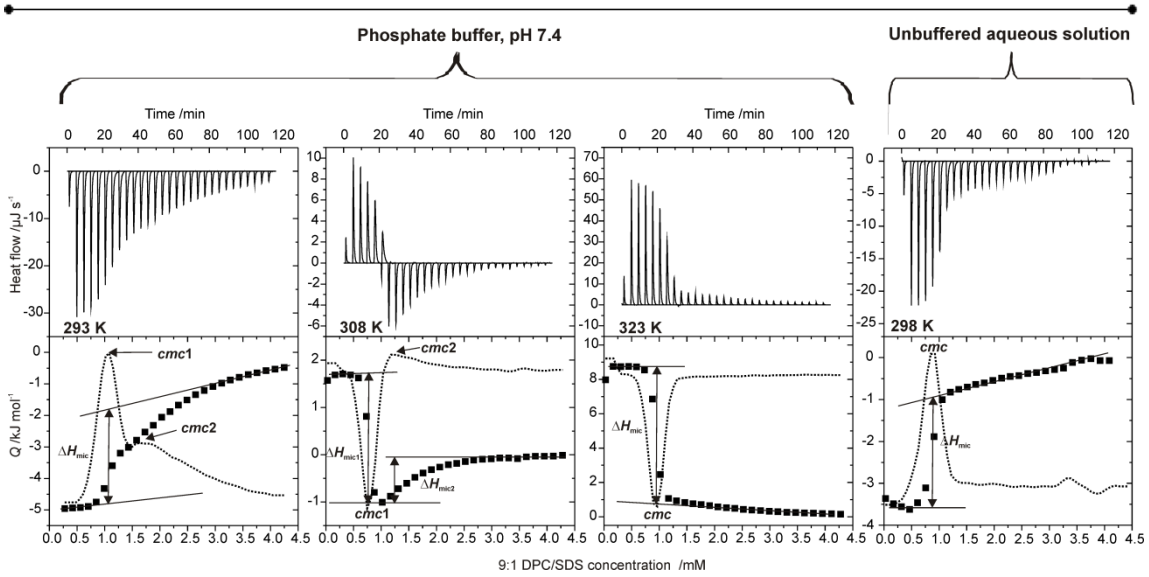


**FIGURE 6**

Thermograms, ITC titration curves and first derivatives of ITC titration curves of the 9:1 DPC/SDS system in phosphate buffer and in unbuffered aqueous solution.

The thermodynamic parameters of micellization processes suggest differences occurring in hydration of the mixed micelles compared to pure ones. The self-diffusion coefficients determined by PFG-NMR experiments indicate that addition of SDS only slightly increases the size of the DPC/SDS micelle. Examination of the data of spin-lattice ($T_1$) and spin-spin ($T_2$) relaxations as well as the $T_1/T_2$ ratios shows that H1 protons (-O$\underline{C^1H_2}$-$CH_2$…$C^{12}H_3$) are the main constituents of the rigid interfacial layer core preventing penetration of water into the hydrophobic interior of a micelle. A comparison of the $T_1/T_2$ ratios along the entire DPC molecule suggests that the hydrocarbon chain of SDS is more or less covered with that of DPC, as expected on the basis of their identical lengths.

A distinct reduction in the transverse relaxation time $T_2$ of the $^{31}P$ nucleus upon raising of SDS concentration compared to small changes observed in the $T_1$ value indicates that addition of SDS to DPC leads to an increase in the overall correlation time, i.e. in the hydrodynamic volume of the micelle.

## CONLCUSIONS

The principal aim of the studies related to the scientific achievement was to establish a relationship between the structure, activity and the interactions with lipid bilayer of the biologically active peptides. The subject area encompassed 21 neurohypophyseal hormone-like peptides and 7 antimicrobial peptides. Owing to the use of the mixed DPC/SDS micelles as a model of biological membrane in conformational analysis of neurohypophyseal hormone analogues, investigations of their dynamics, size and thermodynamic parameters of micellization processes have been also carried out.

Many antimicrobial peptides exist in relatively unstructured conformations prior to interactions with the lipid bilayer. Conformational transition upon binding to the membrane is critical for their function. Therefore, structural studies of antimicrobial peptides are usually carried out in membrane-mimicking environments. Studies on interactions of AMPs with biomembrane models have extensively been continued to either determine or confirm the mechanisms of action. However, with neurohypophyseal hormones, the role of the lipid bilayer as a mediator in the peptide-receptor binding has been ignored. Hence, the studies on interactions of neurohypophyseal hormone-like peptides with lipid bilayer performed within the scientific achievement can be considered as a scientific novelty, because similar investigations of this group of compounds have not been reported to date.

The association of neurohypophyseal hormones and antimicrobial peptides with lipid bilayer involved both electrostatic and hydrophobic interactions. However, there are different final results of these interactions for both groups of peptides due to their different biological functions. The results achieved within the framework of scientific achievement have shown that the modifications of neurohypophyseal hormone analogues with bulky nonpolar residues reduce conformational freedom of the peptides and result in their deeper immersion in the lipid membrane. The N-terminal part of the hormones is the major segment anchoring a peptide to the membrane. The interactions of the neurohypophyseal hormone-like peptides with the membrane can change its structure and at the same time affect the structure and function of GPCRs. Therefore, it is important that they should not lead to destabilisation of the lipid bilayer. In turn, such an effect is appreciable for antimicrobial peptides, acting by permeabilisation of bacterial membranes. Another effect of AMPs interactions with the membrane can also be segregation of membrane lipids, which results in the formation of domains rich in negatively charged or neutral lipids.

The studies carried out within the framework of scientific achievement have a cognitive character. Moreover, the results obtained allow to get a better understanding of the role played by interactions of the peptides with the biological membrane in the activity of peptides which, in turn, can boost the progress in designing new analogues with specific pharmacological properties.

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## **5.** Discussion of other scientific works

### **5.1** Other scientific achievements

My scientific interests focus on conformational studies of peptides by using experimental and theoretical methods and analysis of peptide-lipid interactions in the biological membrane systems. Among the peptide studied, in addition to the peptides described in the first part of self-presentation, i.e. neurohyphophyseal hormones and antimicrobial peptides, are bradykinin analogues, peptides involved in amyloid diseases, peptide modulators of the proteasome activity, peptide imaging agents interacting with chemokine receptor (CXCR4) and transmembrane fragments of bilitranslocase. In addition, in intra-faculty cooperation, I participated in conformational studies of oligomers composed of sugar amino acids and analysis of interactions between biologically active compounds and cyclodextrins. As a result, I co-authored 46 publications included in the Journal Citation Report list (9 of them are scientific achievements),

11 publications in journals not included in the Journal Citation Report list, 4 oral presentations and 40 posters presented at national and international conferences, 1 invited lecture and 1 book chapter (please see **appendix 4b**).

### **5.2** Participation in research projects

In 2003, I received a Ph.D. grant from the Ministry of Science and Higher Education for execution of research in the doctoral thesis. In 2009-2012, I managed the MniSW grant, which has resulted in 9 publications (5 of them are scientific achievements). Moreover, I was an executor of five MNiSW or NCN grants, and now I am an executor of another one. In 2011, I was beneficent of two European projects: East-NMR and Bio-NMR within the 7$^{th}$ Framework Program of EU aiming at the advancement of nuclear magnetic resonance spectroscopy and its applications. These projects enabling me to record NMR spectra in the Slovenian Center of NMR in Lublana, Slovenia.

### **5.3** Scientific cooparation

Experience gained during my research has resulted in establishing national and international scientific cooperations. Previous studies on antimicrobial peptides have been realized in cooperation with Prof. Wojciech Kamysz, the Faculty of Pharmacy with Subfaculty of Laboratory Medicine, Medical University of Gdansk. As part of the international cooperation, I have contributed to conformational analysis of transmembrane fragments of bilitranslocase, an organic anion membrane carrier (Prof. Marjana Novič, National Institute of Chemistry, Lublana, Slovenia, and Igor Zhukov, Ph.D., Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) and conformational analysis of peptide imaging agents interacting with chemokine receptor (Prof. Sridhar Nimmagadda, Johns Hopkins University, Baltimore, United States). This year, I have started cooperation within the studies on inhibitors of the proprotein convertases (Prof. Robert Day, Université de Sherbrooke, Sherbrooke, Canada).