Short thesis for the degree of Doctor of Philosophy (PhD)

Factors influencing the metal ion-catalysed oxidation of the human prion protein (103 – 112) fragment

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Abbreviations

4CF_3O-Salan	2-(((2-((2-hydroxybenzyl)amino)ethyl)amino)methyl)-4-(trifluoromethoxy)phenol
4CF_3O -Salpyran	2-(((2-((pyridin-2-ylmethyl) amino)ethyl)amino)methyl)-4-(trifluoromethoxy)phenol
4F-Salpyran	4-fluoro-2-(((2-((pyridin-2-ylmethyl)amino)ethyl)amino)-methyl)phenol
6F-Salpyran	6-fluoro-2-(((2-((pyridin-2-ylmethyl)amino)ethyl)amino)-methyl)phenol
AA	ascorbic acid
Ac	acetyl group
ACyQ	2-(((2-aminocyclohexyl)amino)methyl)quinolin-8-ol
AEtQ	2-(((2-aminoethyl)amino)methyl)quinolin-8-ol
Ala, A	alanine
Asn, N	asparagine
CD	circular dichroism
CV	cyclic voltammetry
dMKHA	Ac-SKPKTNMKHA-NH_2
dMKHM	Ac-SKPKTNMKHM-NH_2, HuPrP (103-112) fragment
DMSO	dimethyl sulfoxide
di*tb*-Salan	2,4-di-tert-butyl-6-(((2-((2-hydroxybenzyl)amino)ethyl) -amino)methyl)phenol
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His, H	histidine
HPCFUR	pyridine-2-carboxaldehyde 2-furoylhydrazone (E)-N'-(pyridin-2-ylmethylene)furan-2-carbohydrazide
HPCIH	pyridine-2-carboxaldehyde isonicotinoylhydrazone (E)-N'-(pyridin-2-ylmethylene)isonicotinohydrazide
HPLC	high-performance liquid chromatography
HuPrP	human prion protein
Lys, K	lysine
MCO	metal-catalysed oxidation
Met, M	methionine

MetO-Salpyran	4-methoxy-2-(((2-((pyridin-2-ylmethyl)amino)ethyl)-amino)methyl)phenol
MPACs	metal-protein attenuating compounds
MS	mass spectrometry
NH_2	amino group
nMKHA	Ac-SPKTNMKHA-NH_2
Pro, P	proline
ROS	reactive oxygen species
Salan	2,2'-((ethane-1,2-diylbis(azanediyl))-bis(methylene))-diphenol
Salpyran	2-(((2-((pyridin-2-ylmethyl)amino)ethyl)amino)methyl)-phenol
Salquin	2-(((2-((isoquinolin-3-ylmethyl)amino)ethyl)amino)-methyl)phenol
Ser, S	serine
TFA	trifluoroacetic acid
Thr, T	treonine
TOF	time-of-flight analyzator
Trp, W	tryptophan
UV-Vis	ultraviolet visible spectroscopy
Val, V	valine
X1FUR	1-methyl-1H-imidazole-2-carboxaldehyde-2-furanyl hydrazone (E)-N'-((1-methyl-1H-imidazol-2-yl)methylene)furan-2-carbohydrazide
X1THIO	1-methyl-1H-imidazole-2-carboxaldehyde-2-thiophenyl hydrazone (E)-N'-((1-methyl-1H-imidazol-2-yl)methylene)-thiophene-2-carbohydrazide

I. INTRODUCTION AND OBJECTIVES

Metal ions, such as copper(II) and zinc(II), play a crucial role in biological systems, as living organisms rely on inorganic elements for a variety of essential processes. Dysfunction of metalloproteins is associated with a wide variety of diseases, particularly neurodegenerative disorders. The accumulation of metal ions such as copper(II), iron(III), and zinc(II) in specific regions of brain tissue significantly contributes to the development of these disorders. When present in higher-than-normal concentrations, metal ions can promote the onset of neurodegenerative diseases by (i) coordinating with proteins, thus inducing conformational changes and misfolding leading to subsequent aggregation, and (ii) through their metal-catalysed oxidation. These changes contribute to alterations in the tertiary and quaternary structure in the proteins, thereby impacting their functions.

In proteins, the primary targets of reactive oxygen species are the amino acid side chains, which can undergo oxidation. Amino acids prone to oxidation include mainly cysteine, methionine, histidine, and tyrosine. While the oxidation of methionine to methionine sulfoxide is reversible, this is not the case for other amino acids. In biological systems, these processes can be catalysed by redox-active metal ions, which promote the formation of reactive oxygen species. During the metal-catalysed oxidation of biomolecules, the interaction of redox-active metal ions with an electron-donating molecule, such as ascorbic acid, causes the metal ion to be reduced. The reduced form of the metal ion can coordinate with the specific metal-binding sites of biomolecules. It reacts with hydrogen peroxide, forming hydroxyl radicals, which in turn can oxidize neighbouring amino acid side chains. The process is highly dependent on the nature of the adjacent amino acids and the amino acid sequence of the protein. This can lead to the oxidation of the metal-binding site within the protein or peptide. This is mainly a site-specific process in which only one or a few amino acids at the metal-binding sites of the protein are preferentially oxidized. However, cleavage of the peptide backbone is also a possibility. Although the precise role of prion proteins remains unclear, their high methionine content – unusually abundant compared to other proteins – suggests that this unique characteristic may play a significant role in the defence against oxidative stress.

Thus, our objective was to investigate the oxidation of human prion (103 – 112) fragment and the effect of metal-protein attenuating compounds (MPACs) on the oxidation. The study of MPACs was carried out within the framework of two international collaborations. The design, synthesis, and investigation of molecules and chelators that disrupt metal ion-protein interactions for therapeutic purposes commenced several decades ago. Nevertheless, the discovery and development of novel chelators with enhanced properties remain an active area of research. The Bioinorganic Chemistry Research Group has conducted extensive studies on the interaction of peptides with metal ions over many years. Research on the complexation of prion proteins and amyloid β fragments with transition metal ions began twenty years ago, while studies on metal ion-catalysed peptide oxidation began approximately a decade ago.

Our objectives included investigating the metal-catalysed oxidation of the peptide via the human prion protein (105-112) mutant fragment (Ac-SPKTNMKHA-NH_2, denoted as nMKHA) in the metal ion/hydrogen peroxide/ascorbic acid system, specifically in the presence of copper(II) and iron(III) ions. We aimed to identify the products formed under various oxidation conditions. The primary focus was to examine the oxidation of the human prion (103-112) fragment in the presence of MPACs (molecules such as aroyl-hydrazones and salan-type compounds). To achieve this, we explored the coordination chemistry of the MPACs and chelators with copper(II) and zinc(II) ions. Additionally, for two water-soluble aroyl hydrazones, X1FUR and X1THIO, as well as two salan-type compounds, AEtQ and ACyQ, competition studies were conducted with the human prion (103-112) fragment to determine whether the formation of mixed-ligand metal complexes is possible.

II. EXPERIMENTAL CONDITIONS AND METHODS

The peptides, Ac-SPKTNMKHA-NH_2, Ac-SKPKTNMKHA-NH_2 and Ac-SKPKTNMKHM-NH_2, used in the oxidation studies were prepared by the Bioinorganic Chemistry Research Group (Department of Inorganic and Analytical Chemistry, University of Debrecen). The aroyl hydrazone compounds were synthesized by the research group of Nicolas Rey (Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, Brazil), while the modified salan and salan-type compounds were synthesized by the research group of George Kostakis (University of Sussex, Brighton, United Kingdom). The studied compounds were made available to us as part of international collaborations.

pH-potentiometric measurements were carried out to determine the protonation constants of the peptide, Ac-SKPKTNMKHM-NH_2, and the studied chelators, as well as to determine the stability constants of their copper(II) and zinc(II) complexes. The experiments were performed in aqueous and water–DMSO 30:70 (V/V%) media at 25°C and at constant ionic strength of 0.2 mol/dm^3 KCl. The experiments were carried out under continuous stirring, and argon was used to exclude atmospheric oxygen and carbon dioxide. The concentration of ligands in the samples ranged between $1x10^{-3}$ and $2x10^{-3}$ mol/dm^3. The metal ion - ligand ratio varied between 1:2 and 2:3, while in the case of mixed ligand samples the metal – ligand ratio was 1:1. Protonation constants of the peptide and chelators, and the stability constants of their metal complexes were calculated by means of general computational programs (PSEQUAD and SUPERQUAD). Based on the calculated values, the pH-dependent species distribution diagrams of the corresponding systems were plotted using MEDUSA program.

UV-visible spectrophotometric studies were carried out in the systems containing copper(II) ions. The experiments were performed with a Perkin Elmer Lambda 25 type double-beam photometer and a VWR UV-1600 PC type single-beam spectrophotometer in the 200-900 nm wavelength range using 1.000 cm quartz cuvettes. Experimental conditions (metal to ligand ratio, concentration, pH range, ionic strength) were similar to those applied for pH metric measurements.

Circular dichroism spectroscopy studies were performed in the case of copper(II) containing mixed ligand mixtures using a JASCO-810 instrument. The spectra were recorded at room temperature in the wavelength range of 220 - 800 nm at different pH values. The measurements were performed in cuvettes with path lengths of 0.100 and 1.000 cm. Experimental conditions (metal to ligand ratio, concentration, pH range, ionic strength) were similar to those applied for pH metric measurements.

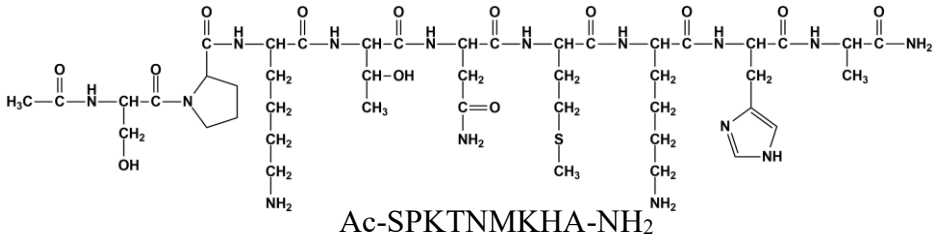
The **oxidation experiments** were monitored using high-performance liquid chromatography (HPLC). The reaction mixtures containing 1.0 mM peptide were incubated at 25 °C for different time periods in the presence of 1% hydrogen peroxide at peptide to H_2O_2 molar ratio 1:4. In the case of nMKHA, the ratios of metal ion and hydrogen peroxide to peptide were varied depending on the studied system. The pH was adjusted to 7.4. The reaction was started by the addition of freshly prepared 1% hydrogen peroxide solution. After incubation, the reaction was stopped by the addition of Na_2EDTA at peptide to Na_2EDTA ratio 1:5. In the case of reaction mixtures containing ascorbic acid, the peptide to ascorbic acid molar ratio was 1:20. The reaction process was monitored by RP-HPLC at different time periods. The samples were analysed by analytical RP-HPLC using a Jasco instrument, equipped with a Jasco MD-2010 plus multiwavelength detector. The oxidized products were analysed using a Teknokroma Europa Protein C18 (250 × 4.6 mm, 300 Å, 5 μm) at a flow rate of 1.00 ml/min, monitoring the absorbance at 222 nm. Mobile phases were water (A) and acetonitrile (B) containing 0.1% TFA. In the case of Cu(II):Ac-SPKTNMKHA-NH_2:H_2O_2:ascorbic acid = 1:1:4:4 system, the oxidized mixture was separated on a semipreparative Grace Vydac Protein and Peptide C18 218TP510 column (250 mm × 10 mm, 300 Å, 5 μm). Reverse phase HPLC was performed on a Jasco instrument, equipped with a Jasco UV- 2077 Plus 4-λ Intelligent UV/Vis detector. The flow rate of 2.00 ml/perc was maintained. The elution of peptides was monitored by UV absorbance at 222 nm.

Mass spectrometry (MS), including LC-MS, offline ESI-MS and MS/MS techniques, were used to identify the oxidized products. The measurements were performed on a Bruker MaXis II. ESI-TOF MS instrument at the Department of Inorganic and Analytical Chemistry (University of Debrecen) by Dr. Csire Gizella, and on a MicroTOF-Q type Qq-TOF MS instrument at the Department of Applied Chemistry (University

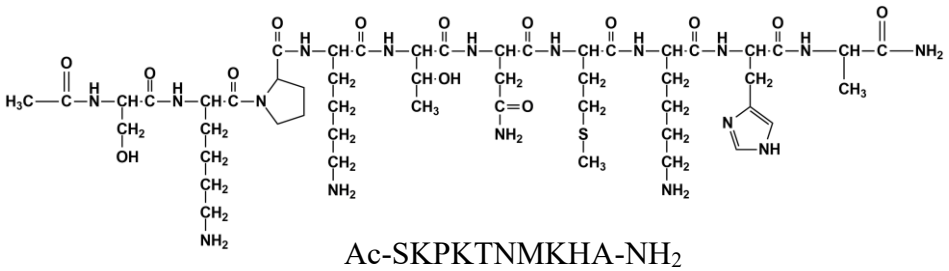
of Debrecen) by Dr. Lajos Nagy. The electrospray ionization (ESI) technique was used as an ion source in positive mode. The MS spectra were evaluated using Bruker DataAnalysis program.

The **cyclic voltammograms** of the copper complexes of X1FUR and Salpyran were recorded using BASI Epsilon EClipse instrument. For the measurement, a carbon working electrode (CHI104), Ag/AgCl 3M NaCl reference electrode, ($E_{1/2}$ = +209 mV vs. NHE) and a platinum counter electrode (ALS Co. Japan) were used in the + 800 – (- 600) mV range. The voltammograms were recorded using a 100 mV/sec scan rate at 25 °C at constant ionic strength of 0.20 M KNO_3. The concentration of ligand in the samples was $1.0x10^{-3}$ mol/dm^3 and the copper(II) to ligand ratio was 1:5.

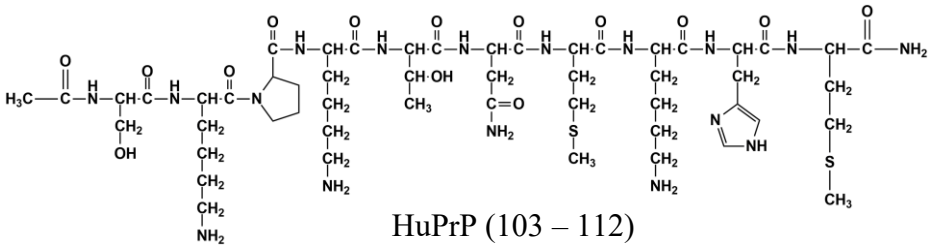
In the case of Salpyran **ascorbate consumption experiments** were performed. This is a simple indirect method to determine whether a compound is able to inhibit the formation of reactive oxygen species. Ascorbic acid as a reducing agent is capable to reduce copper(II) to copper(I), while reactive oxygen species are formed. HEPES buffer at pH 7.1 was used as media. The ascorbic acid consumption was monitored at 265 nm and recorded in 1.000 cm path length quartz cuvette, using an Agilent 8453 spectrophotometer equipped with a Hewlett Packard 89090A thermostat at 25 °C with 300 rpm continuous stirring.



Ac-SPKTNMKHA-NH_2

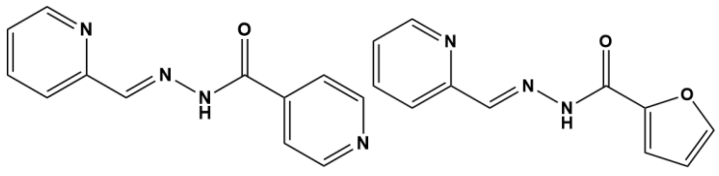


Ac-SKPKTNMKHA-NH_2



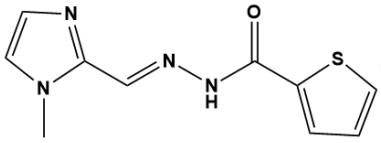
HuPrP (103 – 112)
Ac-SKPKTNMKHM-NH_2

Figure 1. *Structures of the studied peptides*

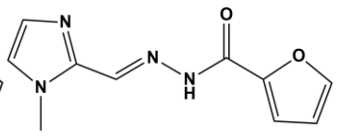


HPCIH

HPCFUR



X1THIO



X1FUR

Figure 2. *Structures of the studied aroyl hydrazones*

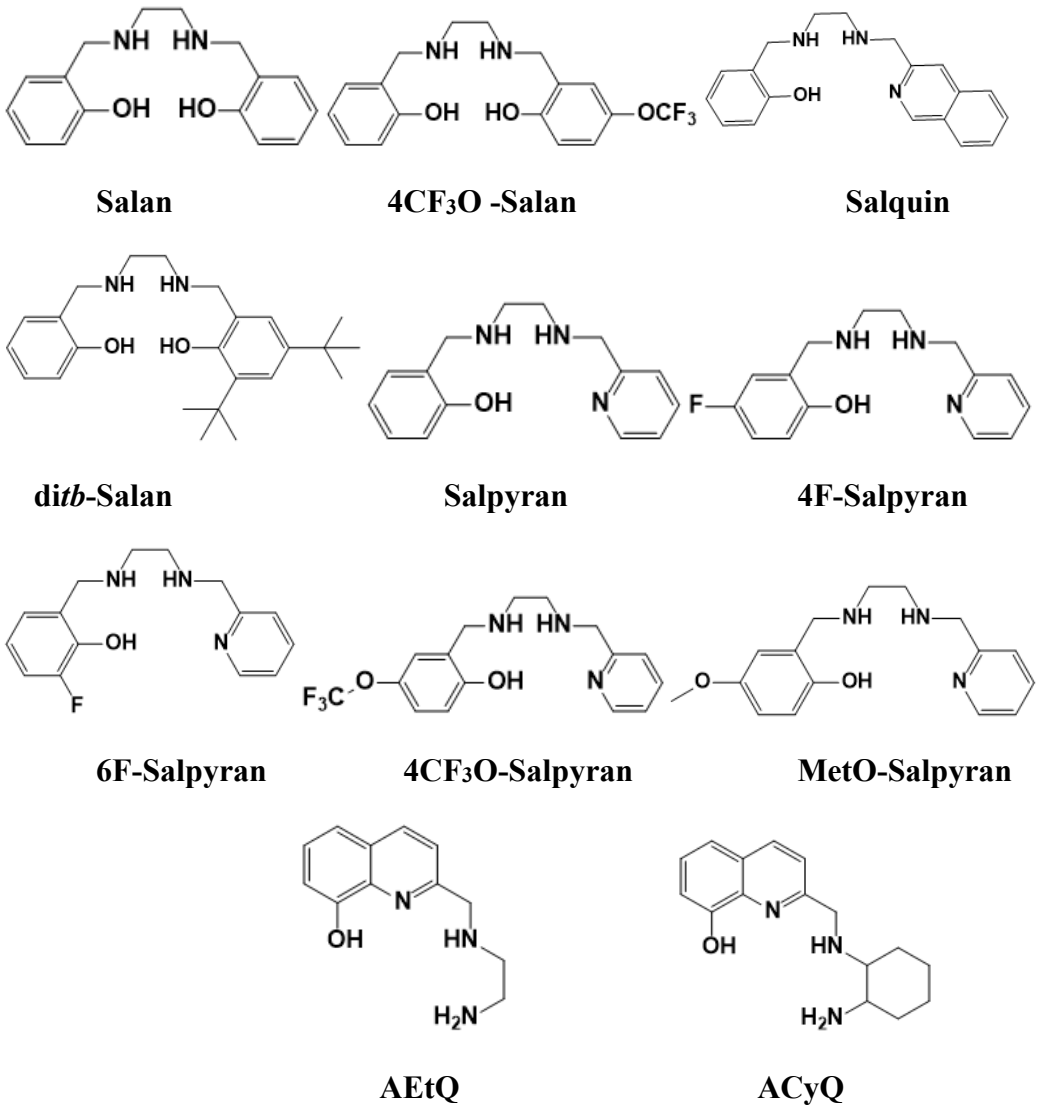


Figure 3. *Structures of the studied salan type ligands*

III. NEW SCIENTIFIC ACHIEVEMENTS

The oxidation of the human prion (105-112) mutant peptide (Ac-SPKTNMKHA-NH_2, denoted as nMKHA) was studied in the presence of Cu(II), Fe(III), hydrogen peroxide, as well as ascorbic acid. Moreover, the oxidation products were also identified.

- We confirmed that primarily methionine is oxidized and the main product formed in every studied system is the methionine sulfoxide derivative (nM(O)KHA). Oxidation of methionine is the most preferred. It occurs only by the action of H_2O_2 and even in the absence of any metal ion. The presence of a redox-active metal ion is not necessary for the oxidation of methionine; however, it catalyses the process.
- If copper(II) ions are present in catalytic amounts or ascorbic acid is also added to the mixture, the oxidation of histidine is also observed. Both the singly oxidized 2-oxo-histidine residue (nMKH(O)A) and the doubly oxidized dioxo-histidine derivative (nMKH(2O)A) products were determined. The simultaneous oxidation of methionine and histidine side chains (nM(O)KH(O)A) was also detected.
- The highest extent of histidine oxidation was detected in the Cu(II):nMKHA:H_2O_2:ascorbic acid = 0.1:1:4:20 system.
- We have found that the formation of an ascorbic acid-nMKHA adduct promotes while the high concentration of copper(II) ions hinders the oxidation of the histidine side chain compared to the reaction mixture containing catalytic amount of copper(II).
- Iron(III) also catalyses the oxidation, although to a lesser extent than copper(II). The difference in the catalytic activity of the two metal ions can be explained by their coordination ability.
- We have ascertained that the addition of ascorbic acid decreases the extent of peptide oxidation, both in the presence or absence of metal ions. However, because of its dual nature, it is not entirely beneficial, as it promotes the irreversible oxidation of histidine versus the reversible oxidation of methionine.

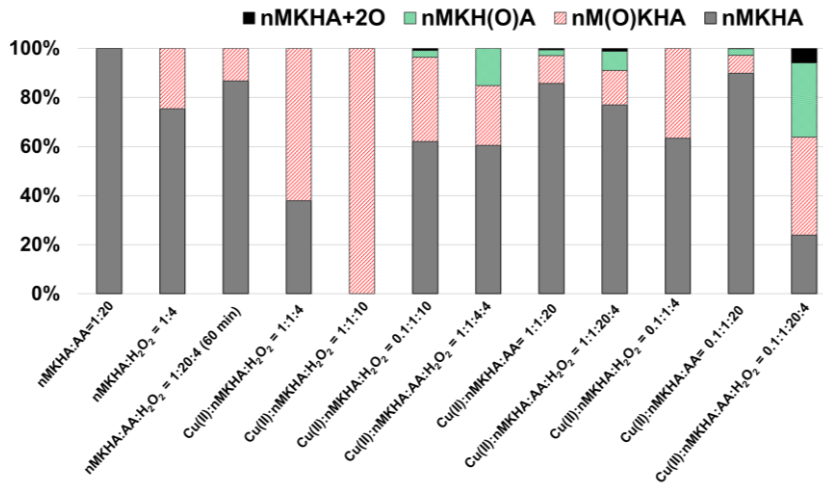


Figure 4. *The ratio of nMKHA to its oxidized products at the 30-minutes sampling point*

In collaboration, we studied the effect of two water-soluble aroyl hydrazones (X1THIO and X1FUR) as metal-protein attenuating compounds (MPACs) on the metal ion-catalysed oxidation of the HuPrP (103-112) fragment (denoted as dMKHM). In order to gain a better understanding of the interactions occurring in solution during the oxidation experiments, we performed solution equilibrium studies in the presence of Cu(II) and Zn(II) ions, and we investigated the complexation process of the prion fragment/aroyl hydrazone mixed ligand systems.

- We have determined that both aroyl hydrazones form five-membered chelate rings with a coordination mode of (N, N, O). The ligands bind to the metal ion through the nitrogen donor atoms of the azomethine and 1-methyl imidazole groups, and the oxygen donor atom of the carbonyl group.
- Both mono- and bis-complex formation can be described; however, with regard to zinc(II) the stability constants are lower than for the copper(II) complexes.
- In the metal ion/HuPrP (103-112) and aroyl hydrazones (X1THIO/X1FUR) ternary systems, the formation of mixed ligand complexes, such as Cu(dMKHM)(X1THIO)H_3, can be

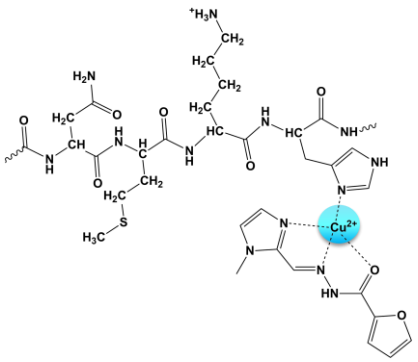


Figure 5. *Structure of the Cu(dMKHM)(X1FUR)H_3 complex*

observed. In the case of X1FUR and X1THIO, the coordination mode of their mixed ligand complexes is similar; the only difference is the deprotonation of lysyl side chains. Copper(II) is bound to the peptide through monodentate coordination to the histidine side chain, while X1THIO/X1FUR occupies the other three equatorial coordination sites with its two nitrogen and one oxygen donor atoms

- Moreover, we successfully determined the Zn(dMKHM)(X1FUR)H mixed ligand complex; its coordination mode is similar to the copper(II) mixed ligand complex, although its stability constant is lower.
- In the oxidation experiments performed at pH = 7.4, the mixed ligand copper(II) complex is the dominant species in the reaction mixtures.
- During the oxidation of the dMKHM peptide in the Cu(II)/H_2O_2/aroyl hydrazone system, the formation of two oxidized products can be observed, – dMKHM + O and dM(O)KHM(O) – in which either one or both methionine is oxidized to methionine sulfoxide.
- Regarding the water-soluble aroyl hydrazones, it can be said that X1FUR slightly suppressed the oxidation and was more promising than X1THIO.
- The poorly water-soluble aroyl hydrazones, HPCIH and HPCFUR, proved to be more effective in suppressing the oxidation.

We studied the oxidation of the M112A mutant of HuPrP (103-112) fragment in the Cu(II)/H_2O_2 system in the presence of aroyl hydrazones X1THIO, HPCIH, and HPCFUR, respectively.

- A slight suppression can be observed in the peptide oxidation even at aroyl hydrazone:dMKHA ratio of 0.1:1; however, this protective effect is more significant at a ratio of 1:1.
- Of the studied aroyl hydrazones, HPCFUR proved to be the most effective; nevertheless, it is unable to inhibit the oxidation of histidine in the presence of ascorbic acid.

In collaboration, we extended our studies to the metal-catalysed oxidation of HuPrP (102–113) in the presence of modified salan-type compounds.

- In the case of the studied molecules, only mononuclear and mono-complexes are formed.
- The methoxy electron-donating substituent influences the stability constant of the formed metal complexes.
- Competition studies were performed with the dMKHM peptide and the compounds AEtQ and ACyQ, respectively. ACyQ does not form a mixed ligand complex with the peptide. In contrast, in the case of AEtQ, whose parent copper(II) complexes have lower stability constants, the formation of several mixed ligand copper(II) complexes and one zinc(II) complex were observed.
- In the case of mixed ligand complexes, the peptide coordinates to the metal ion through the histidine side chain, while AEtQ coordinates through its three nitrogen donor atoms.
- Among the investigated water-soluble salan-type compounds, Salpyran proved to be the most effective in suppressing the oxidation of dMKHM.
- Furthermore, by monitoring the ascorbate consumption, we determined that Salpyran is able to prevent the formation of reactive oxygen species, thus proving itself to be a promising potential therapeutic agent.

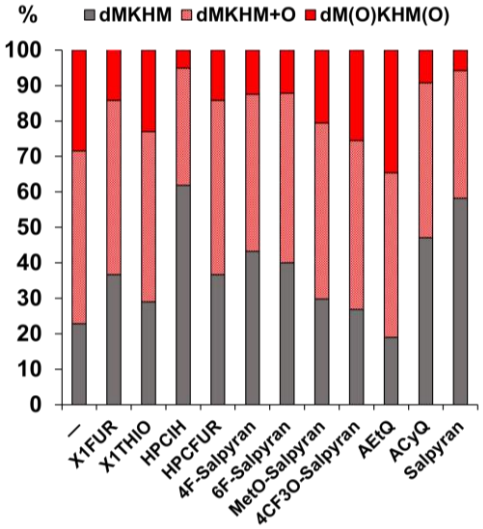


Figure 6. *The ratios of dMKHM and the formed oxidation products at 60-minutes sampling point in the system: Cu(II):dMKHM:H_2O_2:Ligand = 1:1:4:1*

IV. POSSIBLE APPLICATION OF THE RESULTS

The study of metal protein attenuating compounds and chelators and their interaction with biologically relevant metal ions, such as copper(II) and zinc(II), can help to understand which small molecules may be effective in the treatment of certain neurodegenerative diseases and disorders. Their study can facilitate the understanding of oxidation processes in biological systems at the molecular level, both from a coordination chemistry perspective and from a biological perspective – especially by influencing the oxidation processes of biologically relevant molecules. All these results contribute to the future development of metal chelators with more favourable properties, which may be potentially suitable for therapeutic application.

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Registry number: DEENK/375/2025.PL
Subject: PhD Publication List

Candidate: Nikolett Bodnár
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10078483

List of publications related to the dissertation

Foreign language scientific articles in international journals (4)

1. Cukierman, D. S.*, **Bodnár, N.***, Diniz, R., Nagy, L., Kállay, C., Rey, N. A.: Full Equilibrium Picture in Aqueous Binary and Ternary Systems Involving Copper(II), 1-Methylimidazole-Containing Hydrazonic Ligands, and the 103-112 Human Prion Protein Fragment.
Inorg. Chem. 61 (1), 723-737, 2022. ISSN: 0020-1669.
DOI: http://dx.doi.org/10.1021/acs.inorgchem.1c03598
IF: 4.6
* These authors contributed equally to this work.

2. **Bodnár, N.**, Várnagy, K., Nagy, L., Csire, G., Kállay, C.: Ambivalent role of ascorbic acid in the metal-catalyzed oxidation of oligopeptides.
J. Inorg. Biochem. 222, 111510-111519, 2021. ISSN: 0162-0134.
DOI: http://dx.doi.org/10.1016/j.jinorgbio.2021.111510
IF: 4.336

3. Devonport, J., **Bodnár, N.**, McGown, A., Bukar, M. M., Serpell, L. C., Kállay, C., Spencer, J., Kostakis, G. E.: Salpyran: A Cu(II) Selective Chelator with Therapeutic Potential.
Inorg. Chem. 60 (20), 15310-15320, 2021. ISSN: 0020-1669.
DOI: http://dx.doi.org/10.1021/acs.inorgchem.1c01912
IF: 5.436

4. Cukierman, D. S., **Bodnár, N.**, Evangelista, B. N., Nagy, L., Kállay, C., Rey, N. A.: Impact of pyridine-2-carboxaldehyde-derived aroylhydrazones on the copper-catalyzed oxidation of the M112A PrP103-112 mutant fragment.
J. Biol. Inorg. Chem. 24 (8), 1231-1244, 2019. ISSN: 0949-8257.
DOI: http://dx.doi.org/10.1007/s00775-019-01700-2
IF: 3.246

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List of other publications

Foreign language scientific articles in international journals (1)

5. Grenács, Á., **Bodnár, N.**, Pálinkás, D. C., Lihi, N., Várnagy, K.: The effect of side chains on the complex formation processes of N-terminally free hexapeptides containing C-terminal cysteinyl functions.
New J. Chem. 46 (8), 3754-3765, 2022. ISSN: 1144-0546.
DOI: http://dx.doi.org/10.1039/D1NJ05383C
IF: 3.3

Total IF of journals (all publications): 20,918
Total IF of journals (publications related to the dissertation): 17,618

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

10 June, 2025

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *