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In Silico Studies, partition coefficients and tissue permeability of Cu(II)Tripeptide species as Potential Anti-Inflammatory Drugs

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ABSTRACT

Rheumatoid arthritis (RA) is a form of chronic inflammation, a systemic autoimmune disorder that destroys synovial joints. The cause of RA is still unknown, and at present and there is no cure. RA is one of the most common types of chronic inflammatory polyarthritis. It represents a significant health burden with an approximately 1% prevalence worldwide. It mainly affects women, two times more than men. Copper complexes were used in the 1940's to 1970's for the treatment of the inflammation associated with RA. The ligands chosen were sarcosyl-L-histidyl-L-lysine (Sar-His-Lys), sarcosyl-L-lysyl-L-histidine (Sar-Lys-His), sarcosyl-L-histidyl-L-histidine (Sar-His-His), sarcosyl-L-lysyl-L-lysine (Sar-Lys-Lys), sarcosyl-L-glycyl-L-histidine (Sar-Gly-His) and sarcosyl-L-leucyl-phenylalanine (Sar-Leu-Phe). Our previous studies have shown equilibrium constants of H^+, Cu(II), Ni(II) and Zn(II) with these tripeptides and the structures of the complex species were investigated using UV-Vis, ESR and ^{1}H NMR spectroscopy. An objective of the study was to increase the available pool of copper *in vivo*. This was evaluated using a computer model of blood plasma, which considers competition with endogenous metal ions and ligands. It is particularly important that the ligands were evaluated using the Evaluation of Constituent Concentrations in Large Equilibrium (ECCLE) system, an *in vivo* speciation model of blood plasma. Dermal absorption is the preferred method of administration, and so this study used partition coefficients and tissue permeability studies to assess the bioavailability of the different complexes. Based on the results of this study, Sar-Lys-His and Sar-Gly-His have a higher mobilising capacity than all the other tripeptides.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory condition that results in synovial joint destruction (Koopman, William J Moreland, 2005; Sebusi Odisitse & Jackson, 2009; Wang et al., 2007). RA occurs first in the knuckle joints but can affect the synovial membranes of multiple joints in the body(Jackson, Mkhonta-Gama, Voyé, & Kelly, 2000)·(Zvimba & Jackson, 2007b). It is characterised by a massive synovial proliferation and subintimal(M. D. Smith, 2011) infiltration of inflammatory cells, along with angiogenesis(Oklu, Walker, Wicky, & Hesketh, 2010), appears as a slight swelling accompanied by stiffness. RA can cause abnormal tissue growth on bone surfaces(Feldmann, Brennan, & Maini, 1996)·(Koch, 1998). Immunosuppressive drugs generally control the disease, and its symptoms are treated with anti-inflammatory drugs(FREEMAN, 1979) (S Odisitse, Jackson, Govender, Kruger, & Singh, 2007). An effective treatment program for arthritis involves drug therapy (steroidal or non-steroidal drugs), exercise and rest (Weder et al., 2002). There is no cure for RA, but the inflammation associated with this condition has been treated with copper-rich diets such as peanuts, chocolates, shellfish, and certain vegetables(H. T. Delves, 1981). For centuries, copper bracelets have been used as a cure for arthritis. Indeed the amount of copper absorbed from these bracelets has been measured(J. R. J. Sorenson, 1982). Pharmacological evidence suggests that copper complexes can be beneficial in alleviating and treating RA and that these complexes have disease remitting qualities (Forestier, 1945; J. R. J. Sorenson, 1982; Stuhlmeler, 2007).

Lysyl oxidase is a copper-dependent enzyme involved in the renovation of damaged tissue (Harris, 1976). Animal studies have shown that lysyl oxidase activity can be induced with copper (II) sulphate. Alternatively, experimentally induced inflammation is exacerbated by copper deficiency (Frieden, 1986). In humans, metal-tissue distribution studies of copper found elevated serum copper levels during the inflammatory stage of the RA(May, Linder, & Williams, 1977).

While RA is poorly understood, the observed effect of copper on the inflammation associated with the disease provides a basis for drug development and design. Sorenson(J. R. Sorenson, 1976) and Jackson *et al*.(Jackson, May, & Williams, 1978b) showed that Cu(II) complexes are effective in reducing the inflammation associated with RA, the reduction correlating with the dose of Cu(II). They also found the ligands to reduce the toxicity of Cu(II).(Walker, Reeves, Brosnan, & Coleman, 1977)·(Weder et al., 1999) Many ligands have been designed to improve the bioavailability of Cu(II). The first ligands tried were 3,5,9,12-tetraazatetradecanedioic acid (H_2ttda) and 3,6,9-triazaundecanedioic acid (H_2dtda) (Jackson & Kelly, 1989a). Here, the resulting copper(II)–carboxylate complexes were too stable *in vivo* and were excreted intact in urine. Copper is normally excreted in the faeces. In order to change the Cu(II) tissue distribution, ligands with a lower hydrophobicity and reduced Cu(II) thermodynamic stability were tried. Two such ligands, N^1-(2-aminoethyl)-N^2-(pyridin-2-ylmethyl)-ethane-1,2-diamine ([555-N]) and N-(2-(2-aminoethylamino)ethyl)picolinamide ([H(555)-N]), were found to mobilize Cu(II) *in vivo*(Zvimba & Jackson, 2007a). In order to decrease the copper complex stability so that the copper is more labile and hence bioavailable, and at the same time to increase the lipophilicity of the complex, amides were incorporated into the ligand. Because of the hybridisation of the amide nitrogen, it has to deprotonate before it can coordinate to Cu(II). This gives the ligand a negative charge. Using two amides, the resultant Cu(II) complex should be neutral. Also, by burying the charge within the complex, it was hoped that the lipophilicity would improve. For this reason, (1,15-bis(*N*,*N*-dimethyl)-5,11-dioxo-8-(*N*-benzyl)-1,4,8,12,15-pentaazapent-adecane) was synthesised (Nomkoko, Jackson, Nakani, & Hunter, 2006). This ligand formed stable copper complexes, and the adamantine core of the ligand improved the lipophilicity of the complex. In order to test a variety of easily synthesised diamide ligands, use was made of tripeptides. Tripeptides have a range of potential donor atoms, and the complexes formed exist in various stoichiometries (K. Murray and P. M. May, 1984)·(Pettit, Steel, Formicka-Kozlowska, Tatarowski, & Bataille, 1985). The naturally occurring copper transport protein serum albumin has the Asp-Ala-His motif. Jackson *et al.* investigated Sar-His-Lys and Sar-Lys-His and found that these ligands form more stable complexes with Cu(II) than the *in vivo* competitors, Zn(II), Ca(II) and Ni(II). This is an essential factor in the development of copper-based therapeutics (Hammouda, Jackson, Bonomo, & Elmagbari, 2016), as the ligand should not alter the bio-distribution of other metal ions. Using a computer model of plasma, calculations revealed that these tripeptides were not able to mobilise endogenous copper. For this reason, the ligand sarcosyl-L-lysyl-L-lysine(Sar-Lys-Lys) was investigated (Hammouda et al., 2021). While the lysine side chain may increase the hydrophilicity of the copper complex, the side chain amine may also coordinate with the copper, thereby increasing the stability of the complex. At the same time, the terminal sarcosine was retained by the need to increase the lipophilicity of the complex and the biological half-life of the ligand (Pickart et al., 1980), (Pickart, Vasquez-Soltero, & Margolina, 2012).

There are three available routes of administration of copper complexes, oral administration, injection or transdermal administration. Of the three, the transdermal route is preferred as it is the most suitable for long-term therapy. For this reason, the membrane permeability of the Cu(II) tripeptides systems were measured.

Experimental

Materials:

All chemicals and reagents were of analytical grade and were used without any further purification. sarcosyl-L-histidyl-L-lysine (Sar-His-Lys), sarcosyl-L-lysyl-L-histidine (Sar-Lys-His), sarcosyl-L-histidyl-L-histidine (Sar-His-His), sarcosyl-L-lysyl-L-lysine (Sar-Lys-Lys), sarcosyl-L-glycyl-L-histidine (Sar-Gly-His) and sarcosyl-L-leucyl-phenylalanine (Sar-Leu-Phe).) were purchased from GL Biochem (Shanghai) Ltd and its purity was checked by a chromatographic method. The other chemicals were purchased from Sigma and used without further purification. The metal-ion stock solutions were prepared from analytical grade reagents, and their concentration was checked using EDTA titration (Morgan, 1990).

Measurements:

Partition coefficients were measured using the shake flask method; the organic phase was 1-octanol, pre-saturated with water (Leo, Hansch, & Elkins, 1971a),(Xiang & Anderson, 1994), and the aqueous phase was deionised water. Solutions of $CuCl_2$ (0.005 M) and tripeptides (0.005 M) were prepared in distilled/deionised water, and the pH was adjusted to 2.0 to 11.0 using NaOH or HCl. Aliquots (5 ml) of the solutions were pipetted into glass vials and mixed with 6 ml of 1-octanol (99 %). The vials were shaken for two minutes at 298 K. After separating the two phases, the organic phase was back-extracted using 5% HNO_3. The total concentration of copper in each phase was measured at 327.39 nm using an Agilent 4100 Microwave Plasma-Atomic Emission Spectrometer (MP-AES). The detection limit of Cu(II) at this wavelength was 0.2 ppb. Membrane diffusion was measured using a modified Franz cell in which the two cells were horizontal relative to each other and separated by an artificial membrane. The receiver cell was filled with distilled/deionised water, while the donor cell was filled with $[CuL]^+$ at a pH of 7.4. Both cells were covered to prevent evaporation. The entire apparatus maintained at a constant temperature of 298 K. The artificial membrane was made using filter paper submerged in Cerasome 9005, dried for a few minutes at room temperature and then weighed. The amount of lipid

absorbed, determined by mass difference, was 0.085±0.002 g, and the diffusion area between the cells was 0.709 cm^2.

Results and discussion

Blood Plasma Model

The present study was carried out to investigate the *in vivo* Cu(II) speciation of the studied ligands using the program ECCLES (May et al., 1977). This program has a database of some 40 ligands and seven metal ions usually found in blood plasma (Zeevaart et al., 1999). ECCLES calculates the concentration of all the low molecular mass species present in plasma, given the total concentration of the ligand of interest. From this, it is possible to calculate the ligand's plasma-mobilising index (p.m.i.) concerning a particular metal ion. P.m.i. is a measure of the ability of the ligand to increase the low molecular mass concentration of the metal ion of interest. For Cu(II), the plasma mobilising index is defined (Jackson, May, & Williams, 1978a),(May & Williams, 1977) as;

$$\text{p.m.i} = \frac{\text{total concentration of low molecular weight metal complex species in the presence of a drug}}{\text{total concentration of low molecular weight metal complex species in normal plasma}}$$

For any ligand metal system, a high log p.m.i value at low ligand concentration indicates that the ligand is a good competitor against other potential ligands present in the blood plasma (Sebusi Odisitse, Jackson, Govender, Kruger, & Singh, 2007). **Fig.1** shows log p.m.i of different tripeptides in this study complexed with copper as a function of tripeptide concentration.

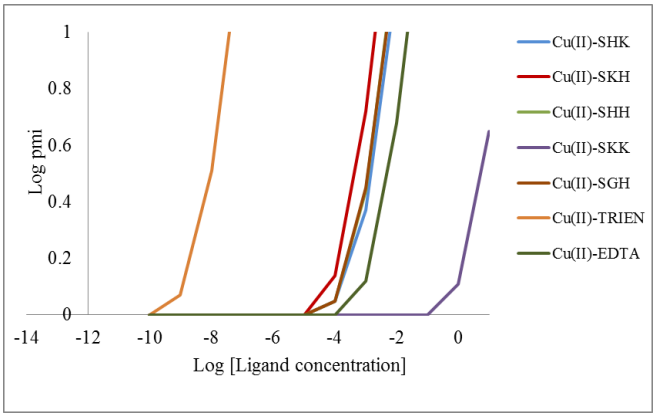

Fig. 1: Plasma mobilising index for Cu(II) with tripeptide and TRIEN complexes.

Sar-Lys-Lys was not able to mobilise copper *in vivo.* The reason for this is the relatively high affinity of Sar-Lys-Lys for Zn(II). The lack of an imidazole group in this ligand means that it is not as selective for copper. With the high *in vivo* concentration of Zn(II), Cu(II) is not able to compete effectively. Although it has been reported that tripeptides containing an imidazole residue are not particularly good at mobilising Cu(II) *in vivo* [(Jackson & Kelly, 1988)], it seems the better mobilisation arising from Sar-Lys-His, Sar-Gly-His, Sar-His-His, Sar-His-Lys as opposed to Sar-Lys-Lys are as a result of the high basicity of the imine nitrogen of the imidazole ring with a reported pK_a value of 6.95 [(Ueda, Miyazaki, Matsushima, & Hanaki, 1996),(Alí-Torres, Rodríguez-Santiago, & Sodupe, 2011)]. However, better mobilisation of Cu(II) as compared to Ni(II), Ca(II), and Zn(II) could be due to the preferential binding of ligands to Cu(II) compared to Ni(II), Ca(II) and Zn(II).

Poor mobilisation of Ni(II) was observed in this study even though Ni(II) formed more stable complexes compared to Zn(II) and Ca(II). *In vivo*, the free concentrations of Ca(II) and Zn(II) are 10^{15} and 10^{9} times greater than the free concentration of Ni(II), and this higher concentration means that they are able to displace Ni(II) from its complexes (Sebusi Odisitse & Jackson, 2014).

The p.m.i curves of different tripeptides and triethylenetetramine TRIEN (Jackson & Kelly, 1989b),(Aaseth, 2012) are shown in Figure 5.6. In comparison, TRIEN is six to three orders of magnitude better at mobilizing Cu(II) *in vivo* than Sar-Lys-His, Sar-Gly-His, Sar-His-His and Sar-His-Lys. The improved mobilizing ability of TRIEN compared to these tripeptides is related to the stable Cu(II) complexes of TRIEN and weak Ca(II) binding. EDTA also shows this, which is a poor mobiliser of Cu(II), which forms very stable complexes. For EDTA, the Ca(II) complex is also very stable.

Octanol/water partition coefficients

Cu(II) complexes can be administered by absorption or by injection either intravenously or intraperitoneally. Since we are aiming for long-term therapy, injection is not a viable option. The metal ion has to cross a lipid barrier (Jackson et al., 1978b). The usefulness of a drug administration procedure depends on the lipophilicity and molecular weight of the drug (Leo, Hansch, & Elkins, 1971b) (Xiang & Anderson, 1994). Traditionally, the lipophilicity of a drug has been estimated by its partition coefficient. For Cu(II), the partition coefficient between octanol and water is defined as:

$$\log P_{oct\backslash aq} = \log\left(\frac{[Cu]_{oct}}{[Cu]_{aq}}\right) \quad (1)$$

where $[Cu(II)]_{oct}$ is the concentration of Cu(II) in the octanol phase and $[Cu(II)]_{aq}$ is the concentration of Cu(II) in the aqueous phase. Since the different Cu(II) complexes will have different partition coefficients, and the Cu(II) speciation is pH dependent, the partition coefficient will also be pH dependent. This study seeks a viable transdermal alternative for the delivery of copper as a therapy for inflammatory disorders, and therefore the degree of lipophilicity is important.

Cu(II) Sar-His-Lys

The Cu(II) Sar-His-Lys results are shown in **Fig 2**, which shows the log $P_{oct\backslash aq}$ results and the speciation graph for Cu(II) Sar-His-Lys as a function of pH. The Cu(II) complexes of Sar-His-Lys were more soluble in water than in 1-octanol, as is shown by the negative values of log $P_{oct\backslash aq}$.

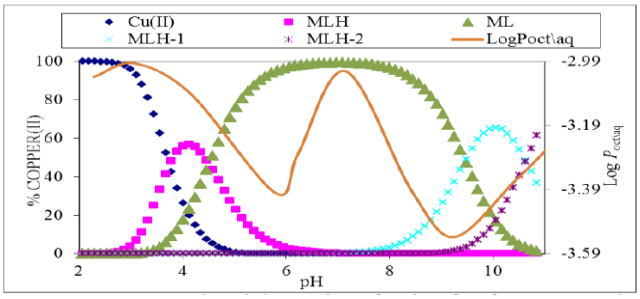

Fig. 2: Log $P_{oct\aq}$ and speciation graph as a function of pH for 1:1 Cu-Sar-His-Lys.

The partition coefficients changed as the pH increased due to the formation of various species. Comparing the speciation diagram and the log $P_{oct\aq}$ curve is interesting as it clearly shows how the partition coefficient changes as the CuL species is formed and transformed into $CuLH_{-1}$. The Log $P_{oct\aq}$ of -3.02 at pH 7.15 can be ascribed to this species.

Cu(II) Sar-Lys-His

Results for Cu(II) Sar-Lys-His are presented in **Fig. 3**. The solubility in 1-octanol increased from low pH to pH 7.07. At physiological pH, $CuLH_{-1}$ was the predominant Cu(II) species at 97 %. At this pH, only 0.88 % of the Cu(II) was extracted into the organic phase of the octanol-water mixture.

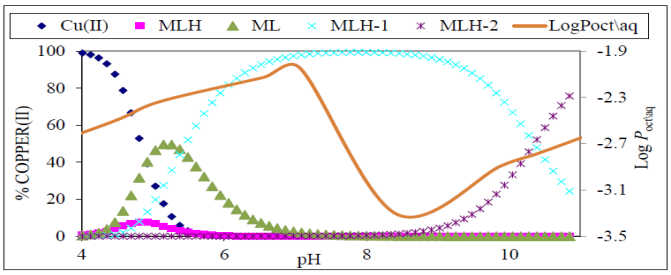

Fig. 3: Log $P_{oct\aq}$ and speciation graph as a function of pH for 1:1 Cu-Sar-Lys-His.

This complex is relatively hydrophilic by having a negative log $P_{oct\aq}$ value. At pH 10.4, $CuLH_{-2}$ was the most predominant species with a log $P_{oct\aq}$ value of ~-2.79.

Cu(II) Sar-His-His

The negative values of log $P_{oct\aq}$ for Cu(II) Sar-His-His show that this complex is largely hydrophilic (**Fig. 4**). The partition coefficient profile is quite complex in that it increases, decreases and then increases again. This profile, however, is easily rationalised by reference to the speciation diagram. At pH 4.13, the most predominant species was CuLH, with a log $P_{oct\aq}$ = -2.43; at pH 7.43 the most predominant species was $CuLH_{-1}$, with a log $P_{oct\aq}$ = -2.9 and at pH 10.05 the most predominant species was $CuLH_{-2}$, with a log $P_{oct\aq}$ = -2.74.

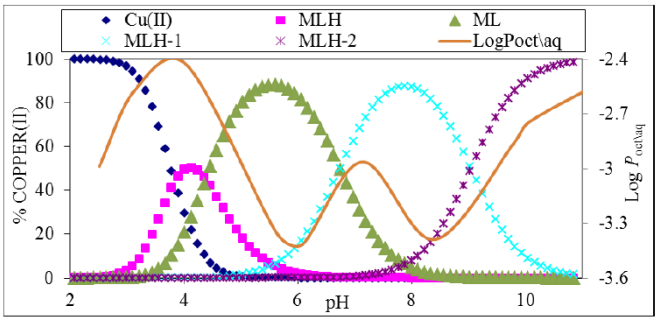

Fig. 4: Log $P_{oct\aq}$ and speciation graph as a function of pH for 1:1 Cu-Sar-His-His.

Cu(II) Sar-Lys-Lys

Results for Cu(II) Sar-Lys-Lys are given in **Fig. 5**. The solubility in 1-octanol increased from low to high pH. There was a rapid increase in log $P_{oct\aq}$ values as the pH increased from 6.15-11.10 due to the formation of CuL (95.67 %) with maximum log $P_{oct\aq}$ values of -2.6. The relative hydrophilicity of this species and the charge distributions explain the preference of the complexes for the aqueous layer resulting in negative values of log $P_{oct\aq}$.

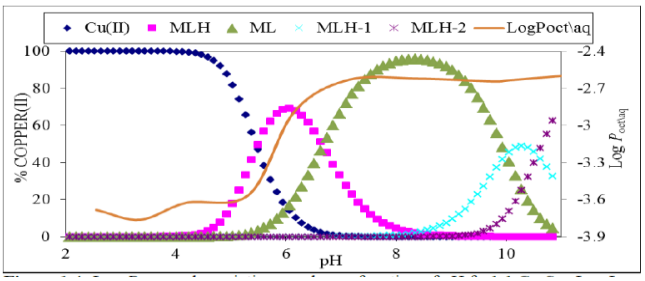

Fig. 5: Log $P_{oct\aq}$ and speciation graph as a function of pH for1:1 Cu-Sar-Lys-Lys.

Cu(II) Sar-Gly-His

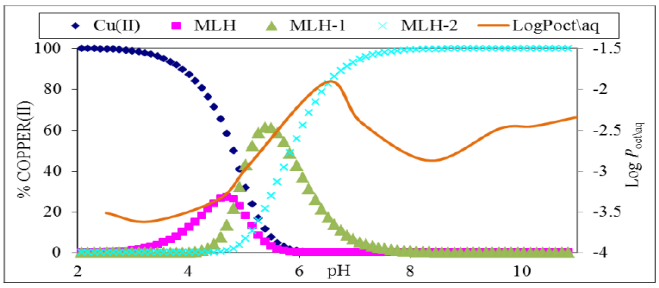

Fig. 6: Log $P_{oct\aq}$ and speciation graph as a function of pH for1:1 Cu-Sar-Gly-His.

The results for Cu(II) Sar-Gly-His are given in **Fig. 6**. The CuLH species was the predominant species at pH 4.56, where the log $P_{oct\aq}$ was -3.34. At pH 6.48, the log $P_{oct\aq}$ values of Cu(II) Sar-Gly-His was -1.91, and the $CuLH_{-2}$ species was predominant at this pH. There was an increase in *log* $P_{oct\aq}$ values from pH 2.52 to 6.48, whereupon it decreased. This partition coefficient profile does not precisely match the species distribution curves. The explanation for these observations is that the MLH_{-2} species

has a higher partition coefficient than MLH_{-1}, although the curve decreased above pH 7 as the concentration of MLH_{-2} increased.

Cu(II) Sar-Leu-Phe

At low pH values, the partition coefficient values for Cu-Sar-Leu-Phe in Figure 1.7 also remain at -3 until a pH of 4.4. From a pH of 4.4 to 6.5, it increases to -1.6, after which the partition coefficient values decrease to -2.2 at the end of the pH range. The overlaid speciation curve for Cu-Sar-Leu-Phe in **Fig. 7** indicates that species only start forming at a pH of 4.2, which corresponds to the increase in partition coefficient values at a pH of 4.4. The speciation curve also indicates that until a pH of 4.8, the increase in partition coefficient is due to the ML species. All the other partition coefficient values are due to a combination of the four possible species (ML, ML_2H_{-1}, MLH_{-1} and MLH_{-2}). At the physiological pH of 7.4, the ML_2H_{-1} species predominates and has a partition coefficient of approximately -1.7.

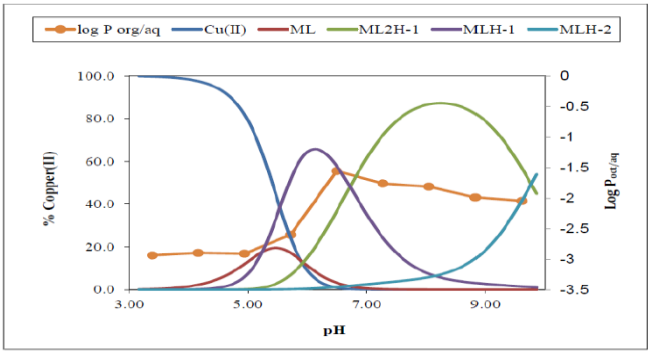

Fig. 7: Log $P_{oct\backslash aq}$ as a function of pH and speciation graph for 1:4 Cu-Sar-Leu-Phe.

Membrane permeability studies.

Dermal absorption of chemicals has been tested in humans, rats, rabbits, pigs and mice (Barbero & Frasch, 2009). The percutaneous absorption of a drug can be expressed as the permeability coefficient (K_p cm/h), which depends on the steady-state flux (J) of the drug across the membrane and applied dose:

$$K_p = \frac{J}{Ci} \qquad (2)$$

where, J is the number of mg/cm^2. h of the permeant crossing the membrane, and Ci is the concentration of the drug in the donor phase. The steady-state flux, J, can be expressed

$$J = \frac{Q}{A.t} \qquad (3)$$

where Q (mg) is the quantity of permeate transported through the membrane in time t (hrs), and A is the exposed membrane area in cm^2. A horizontal Franz cell, fitted with an artificial membrane, Cerasome 9005, was used in the present study. This membrane is a lipid solution, which mimics the human stratum corneum.(Hostynek, Dreher, & Maibach, 2011) Krulikowska *et al* (Krulikowska, Arct, Lucova, Cetner, & Majewski, 2013) have shown that lipid mixtures, similar to those present in the intercellular spaces of the stratum corneum, are valid skin models. These authors found a 95% correlation between the penetration coefficients of porcine skin and Cerasome 9005. For this reason, cerasome has been used in this study. The results are given in **Table 1**. As can be seen, ligands are able to promote the dermal absorption of Cu(II); for Sar-Gly-His, there is a two fold increase relative to copper chloride.

It is interesting to note that, for Cu(II), there is no correlation between partition coefficient, the traditional measure of lipophilicity, and permeability coefficient. Instead, some authors have found a correlation between K_p and complex stability (R. M. Smith & Martell, 1989). Skin permeation is a complex process and depends not only on lipophilicity but also on molecular mass and hydrogen bonding. This indicates that care must be exercised when using partition coefficients as a proxy for tissue permeability.

Table 1: Flux of diffusion J and permeability coefficient K_p of copper tripeptide complexes through Cerasome 9005 membrane at pH 7.0.

Complexes	J mg\cm^{2}h	K_p cm\h
Cu(II)Sar-His-Lys	0.009 ± 0.01	0.049 ± 0.01
Cu(II)Sar-Lys-His	0.013 ± 0.01	0.047 ± 0.01
Cu(II)Sar-His-His	0.012 ± 0.01	0.041 ± 0.01
Cu(II)Sar-Lys-Lys	0.005 ± 0.01	0.038 ± 0.01
Cu(II)Sar-Gly-His	0.007 ± 0.01	0.061 ± 0.01
Cu(II)Sar-Leu-Phe	0.009 ± 0.002	0.206 ± 0.019
$CuCl_2.2H_2O$ (pH 4.2)	0.004 ± 0.05	0.028 ± 0.07

Conclusions

The study's objective was to determine the plasma mobilising capacities and measure the lipophilicity of the metal-ligand complexes. The results obtained from this study were compared with the terminal amino acid sequence of serum albumin, which is the endogenous copper transport protein. Our study demonstrated that the Cu(II)-binding affinity of these ligands are comparable with those of glycyl-glycyl-L-histidine (Gly-Gly-His) and L-aspartyl-L-alanyl-L-histidine N-methyl amide (Asp-Ala-His-NHMe). The present study has contributed to the understanding of some aspects and problems involved in the development of copper complex agents to alleviate inflammation associated with rheumatoid arthritis (RA). At the same time, the tripeptides with histidine demonstrated that the metal ion complexation strongly depends on the position of histidine

in the tripeptide molecules. Besides introducing new ideas that can be successfully applied in solution chemistry, the study has clearly outlined the approach that could be followed in future studies for investigating new anti-inflammatory drugs for RA. Lysine was used in this study, but our results show that its side chain does not coordinate to the Cu(II). Thus it would be better to use a more lipophilic amino acid in future. Furthermore, it would be interesting to investigate the copper bio-distribution in animal experiments using ^{64}Cu(II) as a radiotracer. In addition, *in vivo* experiments using an animal model of inflammation, like Carrageenan foot edema, using the most promising complexes could be investigated as well.

Acknowledgements

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