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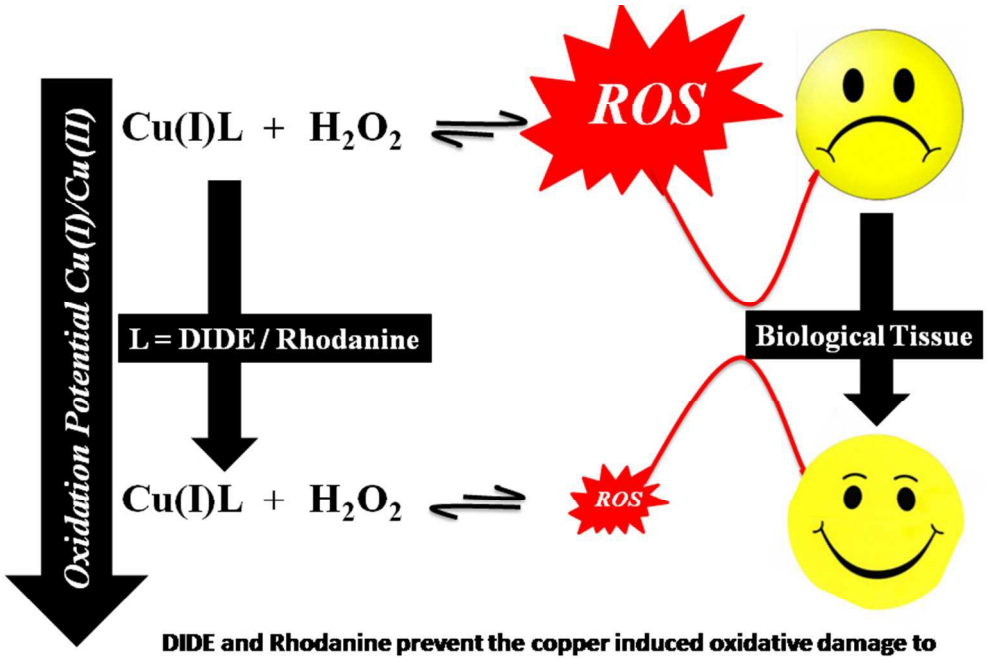

DIDE and Rhodanine prevent the copper induced oxidative damage to proteins by binding copper into redox inactive state

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Targeting Copper Induced Oxidative Damage to Proteins by Ligation: A novel approach towards Chelation Therapy for Oxidative Stress Disorders

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Abstract

Oxidative stress due to excessive accumulation of reactive oxygen species (ROS) triggers the onset of various pathological conditions in humans such as, cancer, arthritis, muscular dystrophy (MD), Alzheimer´s and Parkinson's disease. Till date, the available therapeutic strategies mainly focus on removal or sequestering of excessive ROS rather than preventing their generation. In the present study we hypothesize that the selective ligation of copper and iron ions in their inactive redox states with appropriate ligands can be a novel approach for prevention of free radical generation. To testify this hypothesis the present study was devised to explore the structural, conformational and electronic requirements of a potential biocompatible inhibitor of copper induced oxidative damage to proteins. A series of chemical compounds such as; neocuproine, bathophenanthroline, thiourea, acetonitrile, bathocuproine, dimethylsulfoxide,

mannitol, urea and histidine were tested for their impact on copper induced oxidative damage to proteins. To measure the oxidative damage, bovine serum albumin (BSA) was used as the model substrate to quantitate the conversion of its free amino groups to the carbonyl counterparts under the ROS induced oxidative stress. The observed complexational behavior of Cu with the investigated ligands revealed that a ligand with soft donor site, donor sites placed at the corners of a tetrahedron or a good π-back acceptance character shall exhibit excellent potential to inhibit copper induced oxidative damage to proteins. To validate these facts we tested two bioactive molecules viz. (*1,2-(diimino-4'-antipyrinyl)-1,2 diphenyl ethane* (DIDE) and Rhodanine whose distinctive molecular structure meets the hypothesized prerequisites, for their protective impact against Cu induced free radical-mediated oxidative damage. Expectedly both DIDE and Rhodanine were found to offer excellent protection against copper-catalyzed oxidative damage to BSA. Furthermore, based on the thermodynamic, kinetic and computational investigations, we propose that the observed antioxidant activity of DIDE and Rhodanine is due to the selective stabilization of Cu(I) by these bioactive and biofriendly molecules. To the best of our knowledge, the present study is the first of its kind to establish that the antioxidant activity of DIDE and Rhodanine is an outcome of their ability to reduce the generation of ROS by binding copper into a redox inactive form.

Key Words: Copper Induced Oxidative Damage; Reactive Oxygen Species; Rhodanine; (1,2-(diimino-4'-antipyrinyl)-1,2 diphenyl ethane (DIDE); antioxidant.

Introduction

The living cells under aerobic conditions are threatened by the production of reactive oxygen species (ROS) i.e., $OH^{\bullet}$ and $OOH^{\bullet}$. Under normal conditions it is efficiently counter-balanced by the powerful enzymatic antioxidant mechanisms of the cell [1-6]. However, the loss of balance between ROS generation and its clearance results in excessive accumulation of ROS in the cells leading to oxidative stress. Under these conditions, the ROS cause the oxidative damage to biological molecules such as nucleic acids, lipids and proteins. The accumulation of these biomolecular injuries results in the onset of a variety of pathological conditions, mostly related to aging, for example, arthritis, muscular dystrophy, cancer and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [7-9]. The excessive generation of ROS does attack vital biomolecules like DNA and RNA leading to the genomic instability. Oxidative stress has been found to be responsible for an estimated 10,000 DNA modifications per cell per day [10]. The oxidative damage on lipids leads to the loss of integrity of biomembranes of cell and its organelles such as mitochondria due to lipid peroxidation. On the contrary the oxidative damage of proteins by ROS was previously considered to be the lesser evil for the cell due to rapid turnover of proteins. Recently, it has been reported that the free radical-induced protein damage could contribute to many disorders [11]. Lately, many studies have been reported which clearly establish that oxidatively modified proteins accumulate during ageing and in some pathological conditions like Alzheimer's and Parkinson's diseases [12, 13].

The classical curative approach for the treatment of oxidative stress-induced cellular damage and resultant disorders are mainly based on sequestering the free radical species by means of antioxidants, thereby limiting their availability for causing biomolecular injuries. In addition to this, an alternative preventive approach to alleviate oxidative stress may involve the use of

potential inhibitors that can reduce the generation of ROS at the onset. In fact, the prevention of free radical generation in the first place should be more effective than their neutralization with antioxidants. Such novel approaches for reducing the generation of ROS is likely more important for risk prevention of oxidative stress in general, and ageing disorders in particular, because with advancing age many of the endogenous antioxidant defense systems fail to offer appropriate protection against elevated oxidative stress. In view of this, the searching and/or designing of potential antioxidants which can inhibit the generation of ROS is one of the most promising area of metalo-biochemistry research.

One of the primary and particularly important source of ROS generation inside the cell are Fenton reactions catalyzed by the redox-active metal ions (like Cu and Fe) (Scheme 1) [14, 15], which accelerate the generation of highly damaging $OH^{\bullet}$ and $OOH^{\bullet}$ radicals from H_2O_2 as substrate (Scheme 1). This excessive generation of ROS modifies the proteins primarily through oxidation of their free amino groups of the N-terminus and the side chains of Asparagine, Glutamine, and Arginine to hydroxyl or carbonyl counterparts, and further by shearing off the peptide bonds [16, 17]. These oxidative modifications of protein molecules lead to their increased recognition and subsequent degradation by cellular protease system, thereby reducing protein functionality and significantly contributing to the loss housekeeping and stress-response enzymatic activities of the living cell [16, 17].

$$Cu(L)_X^{1+} + H_2O_2 \rightarrow Cu(L)_X^{2+} + OH^{\bullet} + OH^-$$

$$Cu(L)_X^{1+} + H_2O_2 \rightarrow Cu(L)_X H_2O_2$$

$$Cu(L)_X^{1+} + H_2O_2 \rightarrow Cu(L)_X^{3+} + 2OH^-$$

Scheme1: Copper catalyzed reduction of peroxide for the generation of ROS.

Since the component reaction steps of Scheme 1 are redox driven, it is possible that the ligands with an ability to ligate with Cu under biological conditions can be potentially employed to prevent the copper induced generation of free radicals, thereby reducing the oxidative damage to living organisms. In prospect of these possibilities and opportunities thereof, we carried out detailed investigations on the impact of ligation characteristics of neocuproine, bathophenanthroline, thiourea, acetonitrile, bathocuproine, DMSO, mannitol, urea and histidine on the copper induced oxidative damage using Bovine Serum Albumin (BSA) as a model protein. The observed activity data was analyzed in light of the complexation behavior of tested ligands with copper to point out the structural and conformational requirements of the potential ligands which can inhibit the copper induced oxidative damage. The resulting correlation analysis indicated that a ligand with soft donor site, and donor sites placed at the corners of a tetrahedron or a good π-back acceptance character shall exhibit excellent potential to inhibit copper induced oxidative damage to proteins. To validate our hypothesis, two pharmacologically important and biologically active ligands viz. 1,2-(diimino-4'-antipyrinyl)-1,2 diphenyl ethane (DIDE) and Rhodanine were selected for investigation. Based on our screening analysis, both these molecules fulfill all the hypothesized requisite structural and conformational requirements of a potential ligand. To verify this, their inhibitory influence on Cu-induced BSA oxidation was studied experimentally. Upon the analysis of subsequent results, it was clearly established that both DIDE and Rhodanine exhibit excellent protection against copper-catalyzed oxidative damage of the tested protein.

The activity results presented in detail in later sections are discussed in light of the structural and electronic properties of the explored ligands. The novelty of the present study lies in the fact that we have for the first time screened out the structural and conformational requirements of the

potential ligands for metal-ion complexation based anti-oxidant activity. This should serve as an ideal platform for the design and development of bioactive ligands with potential to inhibit Cu-induced oxidative damage to the cellular components.

To the best of our knowledge, the presented study is the first report to establish that the antioxidant activity of DIDE and Rhodanine is an outcome of their ability to bind copper into a redox inactive sate. Their selective binding ability for Cu(I) endows them with potential to reduce the generation of ROS. This finding is an important contribution towards mechanistic aspects associated with the promising pharmacological applications of DIDE and Rhodanine. These studies may help to envisage the new and novel therapeutic strategies for the treatment of human oxidative-stress based disorders such as, cancer, arthritis, muscular dystrophy (MD), Alzheimer´s and Parkinson's disease.

Experimental:

Materials

Bovine serum albumin (BSA), 1,10 phenanthroline (phen), histidine, dimethyl sulfoxide (DMSO), neocuproine (NC), bathophenanthroline (Bphen), bathocuproine disulfonate (BC), 2,4-dinitrophenylhydrazine (DNPH), gaunidene hydrochloride, trichloroacetic acid (TCA) and hydrogen peroxide were purchased from Sigma-Aldrich and used as such without further purification

Methods

Protein Carbonyl Formation:

BSA (1 mg/mL) was oxidized with Cu(II)/Ascorbate and Cu(II) / H_2O_2 [0.1 mM Cu(II), 1mM ascorbate, and 1 mM H_2O_2] in 0.1 N phosphate buffer (pH7.4) at 37 °C for 1 h. Protein carbonyls were assayed as described by Levine et al. [18]. Briefly, 1 mL of BSA solution was mixed with 0.5 mL of 10 mM DNPH in 2 N HCl. The mixture was incubated at room temperature for 1 h, followed by the addition of 0.5 mL of 20% trichloroacetic acid. The sample was incubated on ice for 10 min and centrifuged in a bench top centrifuge at 3000*g* for 10 min. The protein pellet was washed three times with 3mL of an ethanol/ethyl acetate mixture (1:1, v/v) and dissolved in 1mL of 6 N guanidine (pH 2.3). The peak absorbance at 370 nm was measured to quantify protein carbonyls. Data were expressed as nanomoles of carbonyl groups per milligram of protein, using a molar absorption coefficient of 22000 M^{-1} cm^{-1} for the DNPH derivatives [18].

Redox activity of copper

Effect of rhodanine and DIDE on the catalytic redox activity of copper was observed by measuring the rate of copper-enhanced oxidation of ascorbate in presence of varying concentration of these ligand species. 10 μM ascorbate in 0.1 M phosphate buffer (pH 7.4), was incubated at 37 °C. Cu(II) (1 μM) were added to this and the absorbance at 265 nm was monitored spectrophotometrically using Shimadzu UV 3600. Same kinetic profile was repeated in presence of varying concentrations (1-4 μM for DIDE and 1-8μM for Rhodanine) of the two ligand species.

UV- visible spectral analysis:

Competition between DIDE and histidine or 1,10- phenanthroline to bind copper ions was determined on the basis of UV- visible spectral analysis over spectrophotometer Shimadzu UV 3600. Cu(II)(histidine)$_2$ and Cu(II)(1,10-phenanthroline)$_2$ complexes were prepared by mixing Cu(II) with either histidine or 1,10- phenanthroline at a 1:2 molar ratio. Reduction of Cu(II) to

Cu(I) was accomplished by adding an excess amount of ascorbate and the spectra were recorded between 400 and 800 nm at room temperature.

Synthesis and characterization of DIDE

The tetra-dentate Schiff base ligand 1,2-(diimino-4'-antipyrinyl)-1,2 diphenyl ethane (DIDE) was synthesized by the refluxing a mixture of 4-Aminoantipyrine and Benzil (2:1) in ethanol at 60 °C for a period of 5 hrs. Sharp increase in melting point coupled with the UV-visible and HPLC data indicated formation of product. Ligand was separated/purified from the unreacted reactants by chromatographic separation over an alumina column. Structural characterization of the ligand was done by the spectral (IR, NMR and Mass) analysis.

Computational analysis

Structural optimization of DIDE in free and complexed state was performed using the Gaussian 03 quantum chemistry package [19]. The initial geometries were optimized by DFT level of theory using Becke's three parameter hybrid functional (B3LYP) [20, 21] and the 6-31G (d, p) basis set. Frequency analysis was performed on the optimized structure at the same level of theory and no imaginary frequencies were found.

Results and discussion

Reactive oxygen species (ROS) are known to convert amino groups of proteins to carbonyl moieties [22, 23], thus the oxidation of proteins results in the formation of carbonyl groups in quantities that reflect the extent of the oxidative damage to the protein. In this context, copper-mediated oxidative damage to BSA was measured by protein carbonyl formation which was quantified experimentally by measuring the absorbance changes at 370 nm. Estimations based on procedures proposed by Levine et al [18], established that incubation of BSA (1mg/ml) for 1hr.

at physiological conditions (37 °C and pH 7.4) with Cu(II)/ascorbate, and Cu(II)/H_2O_2, [Cu(II), 0.1 mM; ascorbate, 1 mM; H_2O_2, 1 mM] led to the formation of 47.5 and 25.7(nmol/mg BSA) protein carbonyls, respectively. A significant decrease in the protein carbonyl content for the same system was observed in presence of some of the ligand species used in present investigation (Figure1).

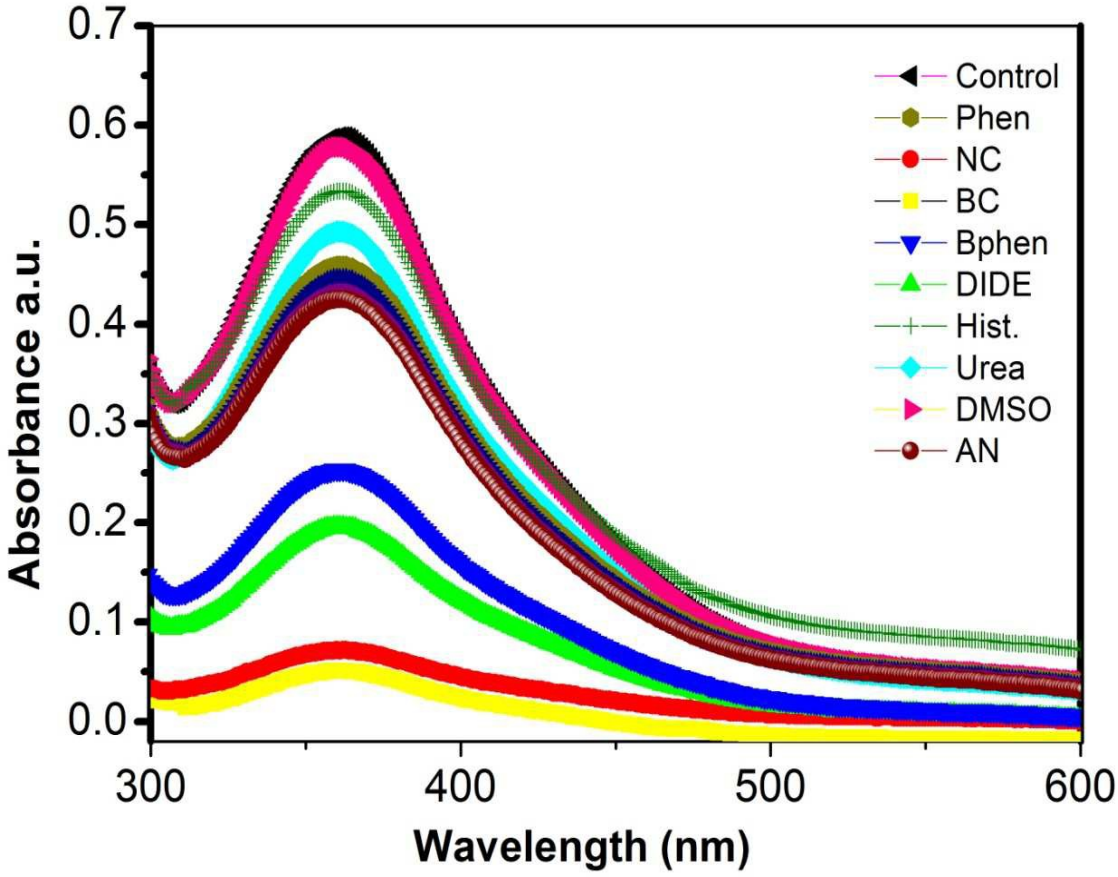


Figure1: Absorbance spectra corresponding to protein-DNPH derivative resulting from oxidative damage of BSA (1 mg/mL incubated for one hour) with Cu(II)/Ascorbate [0.1 mM Cu(II), 1mM ascorbate] in 0.1 N phosphate buffer (pH7.4) at 37 °C in presence of various ligand species.

The inhibition extent quantified as the percent inhibition in comparison to the carbonyl formation in control systems (without ligand species) for the explored ligands are enlisted in Table1.

Table1: Effects of Copper-chelating ligands on Protein Carbonyl Formation (nmol/mg BSA) Induced by the Cu (II)/Ascorbate, and Cu(II)/H_2O_2

Oxidation System		Cu(II)/Ascorbate		Cu(II)/H_2O_2	
Compound	*Conc. (mM)*	*Carbonyls (nmol/mg BSA)*	*Inhibition (%)*	*Carbonyls (nmol/mg BSA)*	*Inhibition (%)*
Control	-	47.5	-	25.7	-
1,10-Phenanthroline	1	19.6	58.8	10.6	58.8
Neocuproine	1	4.1	91.4	2.3	91.1
Bathocuproine	1	2.2	95.4	1.2	95.3
Bathophenanthroline	1	13.4	71.8	7.2	72.0
Urea	1	44.8	5.7	23.8	5.3
Thiourea	1	21.1	55.6	11.4	55.7
Acetonitrile	100	34.1	28.2	18.5	28.0
DMSO	100	47.1	0.8	25.5	0.8
Histidine	1	46.4	2.3	25.1	2.4
Rhodanine	1	19.3	59.6	10.7	58.2
DIDE	1	10.7	77.5	4.4	82.1

Reactions were carried out in 0.1 M phosphate buffer, pH 7.4, at 37 °C for 1 h. Concentrations used were: BSA, 1 mg/ml; Cu(II), 0.1, mM; ascorbate, 1 mM; and H_2O_2, 1 mM. Each value represents the mean of three separate experiments, with a SD of less than 5%

A comparison of the antioxidant activity of the ligands [Table 1], in light of the reported behaviour of the same for their hydroxyl radical scavenging activity [24, 25,26], reveals that the two activities are not correlated. While DMSO does not inhibit the protein oxidation, presence of BC, Bphen and NC which possess copper chelating potential was found to significantly inhibit the copper induced damage to BSA. This is suggestive of the fact that the observed antioxidant activity for the investigated ligands involves a mechanism other than $OH^{\bullet}$ radical trapping or scavenging. Reported copper chelating characteristics of the species like Bphen, BC and NC that were observed to exhibit strong inhibitory impact on protein carbonyl formation in present study indicate that probably the observed results [table 1] are an outcome of the differential abilities of the tested ligands for binding the copper to redox inactive state. It is pertinent to mention that BSA has specific high-affinity copper binding sites and the interaction between the two is expected to render copper redox-inactive. Keeping in view the molar concentrations of the BSA and Cu(II) as used in the present work, the BSA-Copper interaction is expected to have negligible impact on the overall observations and conclusions made for the investigated copper induced oxidative damage of BSA [25,26]. All these facts imply that perhaps the observed decrease in the protein carbonyl content for BSA- Copper/Ascorbate system in presence of some of the ligand species is on account of their ability to bind copper into a redox inactive state.

Studies on complexation behaviour of these ligand species with copper (as Cu(I) & Cu(II)) indicate that the oxidative damage inhibiting power of these ligand species has a direct correlation with their higher binding affinity for copper in reduced state (Cu(I)) in comparison to oxidised state (Cu(II)). This enhanced affinity for Cu(I) in complexed state is expected to drag the equilibrium pertaining to ROS generating reactions (Scheme1) more towards reactant side and hence minimizing the generation of ROS. Hence it can be safely argued that the observed

inhibition operates through the modification of redox behaviour of copper on account of the differential binding affinity of the explored ligands for Cu(II) and Cu(I).

This modification in redox behaviour in turn will be accompanied by a change in the reduction potential of Cu(II)/Cu(I) redox couple which is related to the stability constants of resulting complexes with two oxidation states by eq.1 [27].

$$E_{Complex} = E^{0}_{aqua} - (59.16mV)log\frac{\beta^{II}}{\beta^{I}} \quad \text{---------- eq. 1}$$

Where $E_{Complex}$ is the reduction potential of Cu(II)/Cu(I) in complexed form, E^{0}_{aqua} is standard reduction potential of Cu(II) as aqua complex, β^{II} and β^{I} are the stability constants of oxidized and reduced form of Cu(II) with the corresponding ligand species respectively. This modified redox behaviour of copper in complexed state has a direct implication on its participation in the catalytic reactions responsible for ROS generation. In this context the structure and electronic properties of ligand species are expected to play an important role in controlling the copper induced oxidative damage to the proteins. Hence the inhibition activities of the ligands were analysed in light of their structural and electronic characteristics so as to point out the functional and conformational characteristics of an ideal ligand with desired inhibition potential.

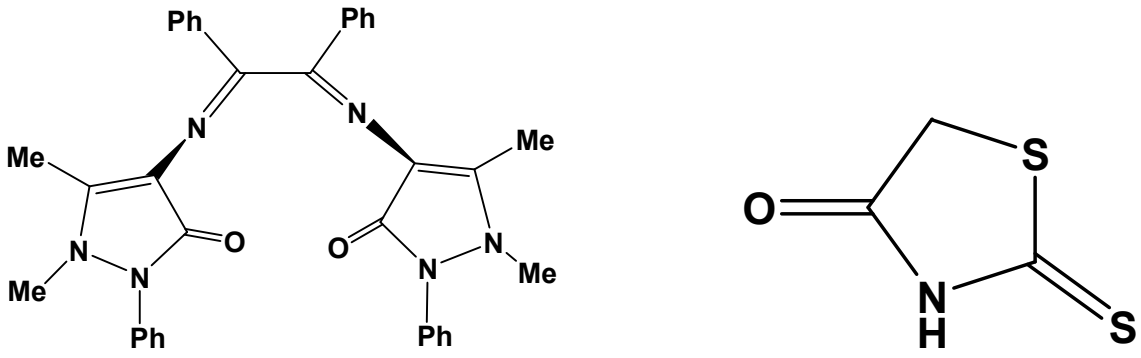
In comparison to phen, NC showed 55% increase in the protein oxidation inhibition (phen.(58.8%) & NC(91.4%)). This enhancement can be attributed to the presence of two extra methyl substituents at *ortho* positions in NC. Their presence leads to a twist in the orientation caused by the steric repulsion of four methyl groups on two NC ligands during complexation with copper. As a result of this steric restriction, tetrahedral geometry is preferred over the square planar geometry. Since the tetrahedral geometry binds Cu(I) preferably over Cu(II) for

crystal field stabilization energy (CFSE) gain, this preferential binding of Cu(I) results in increase in the reduction potential for the Cu(II)/Cu(I) couple [28]. The increased reduction potential in turn restricts the participation of copper for generation of ROS through scheme1.

Similarly the oxidative damage inhibition results for urea and thiourea are suggestive of the fact that the ligand (thiourea) with soft donor atom (S) exhibits a higher inhibition of copper catalysed oxidative damage to the proteins. The observation is in accordance with the predictions of hard soft acid base (HSAB) concept for binding behaviour of ligands with the different oxidation states of metal ions. Since the soft donor site in thiourea will show higher affinity for Cu(I) than Cu(II) (soft-soft interaction), its presence will lead to an increases in the reduction potential of Cu(II)/Cu(I) redox couple, that will restrict the participation of copper in ROS generation and accordingly oxidative damage inhibition is enhanced. BSA oxidation inhibition in presence of NC (91.4%) was observed to enhance further by the attachment of two phenyl rings on NC (BNC (95.3%)). The observation can be attributed to the fact that extended conjugation in BNC enables the ligand (BNC) to show higher pi back acceptance character. As the π-back acceptance character of ligand favourably stabilizes Cu(I) over Cu(II) the reduction potential of Cu(II)/Cu(I) redox couple increases and the participation of copper in ROS generation is minimised.

Based on these observations, it can be concluded that a ligand, with soft donor sites placed at the corners of tetrahedron, or donor sites with good π-back acceptance character shall exhibit excellent potential to reduce copper induced oxidative damage to the proteins. These types of features are available in the molecular structures of two bioactive molecules viz. DIDE and Rhodanine. With a terminal S atom, the Rhodanine molecule is expected to show a similar behavior as that of thiourea and thus expected to show a significant inhibition to the copper

induced oxidative damage to BSA. Similarly in view of our above cited observations we expected that on account of its unique structural characteristics, Schiff base ligand DIDE; derived from 4-aminoantipyrene and benzyl, which is a drug well known for its antimicrobial and antihelminthic activity [29] shall exhibit significant inhibition to the copper induced oxidative damage. Keeping this in view we investigated the protein oxidative damage inhibition properties of both the two bioactive molecules viz. DIDE and Rhodanine.



a) b)

Molecular structure of a) DIDE and b) Rhodanine

The already established biofriendly and bioactive features of DIDE and Rhodanine [29-31] further motivated us to explore them for their protein oxidative damage inhibition potential. We observed that Rhodanine, exhibited appreciable (59.6%) inhibition to the copper induced oxidative damage to BSA. This we attribute to presence of soft donor atom (S) in its molecular structure as in thiourea. To the best of our knowledge, this is the first report of its kind wherein antioxidant property of rhodanine has been observed. DIDE was also observed to exhibit an appreciable (77.4%) inhibition for the copper induced oxidative damage inhibition to BSA (Figure 2).

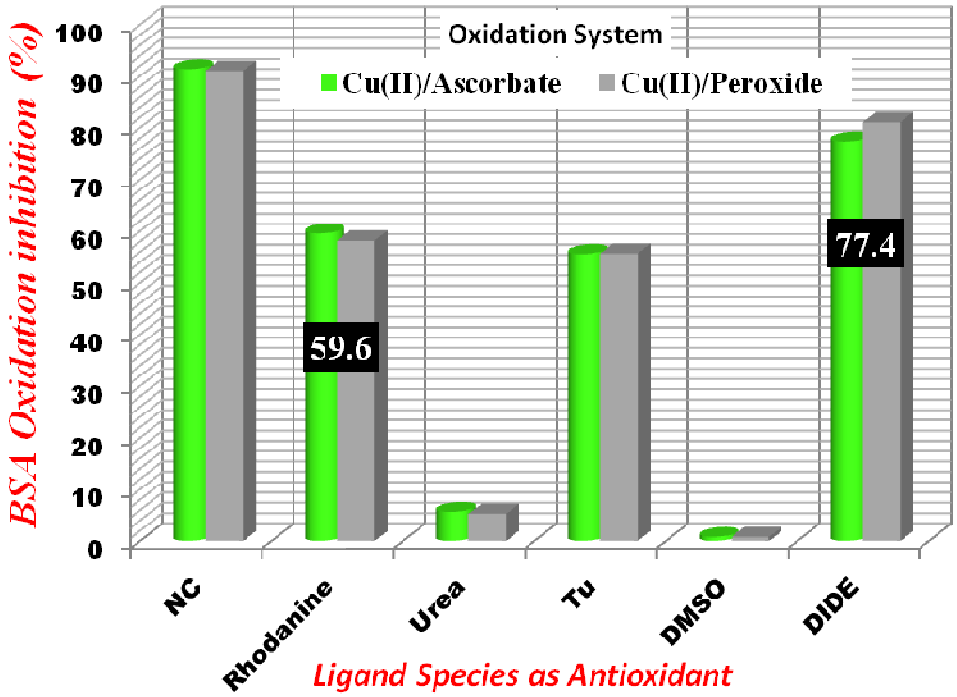


Figure 2: Bar graph representation for BSA oxidation inhibition by Rhodanine and DIDE along with a comparison with some previously reported ligand species.

The BSA oxidation inhibition potential of both the molecules on prima facie can be attributed to their ability to form a stable complex with copper in reduced state (Cu(I)). Such complexation is expected to increase the reduction potential of Cu(II)/Cu(I) couple that in turn is responsible for their BSA oxidation inhibition potential. This presumption was further validated by some additional thermodynamic and kinetic investigations.

Higher binding affinity of DIDE and rhodanine for Cu(I) over Cu(II) was further verified using a model copper complexes, $[Cu(histidine)_2]^{2+}$. It is known that histidine binds Cu(II) in a redox-active manner to form an intensely blue $[Cu(histidine)_2]^{2+}$ complex with λ_{max} 640 nm [32].

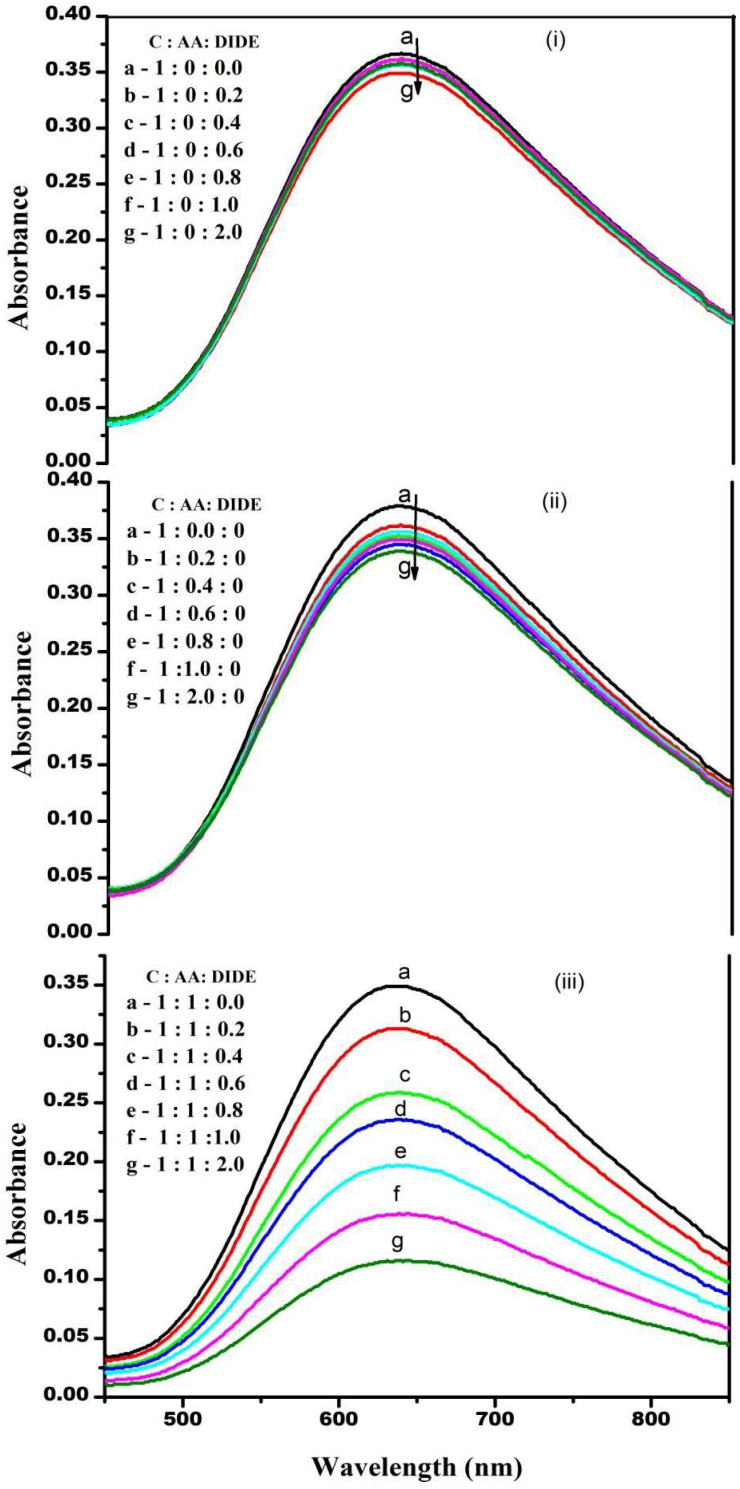


Figure 3: Absorbance spectra of Cu(histidine)$_2$ complex in presence of varying concentrations of (i) DIDE (ii) AA (iii) DIDE + AA.

Addition of DIDE (0-2 mM) and rhodanine (0-4mM) to the $[Cu(histidine)_2]^{2+}$ complex (2mM) led to a negligible decrease in absorbance at 640 nm (Figure 3(i)-(ii)), suggesting that DIDE and rhodanine cannot compete effectively with histidine for Cu(II). In contrast, in the presence of ascorbate (10 mM), addition of DIDE and rhodanine (2 & 4mM respectively) led to a decrease in the absorbance at 640 nm (Figure 3(iii)). These results clearly indicated that DIDE and rhodanine compete effectively with histidine for Cu(I). Similar experiments with $[Cu(phen)_2]^{1+}$, which was produced by mixing $[Cu(phen)_2]^{2+}$ with ascorbate, showed that rhodanine and DIDE compete effectively with 1,10-phenanthroline for Cu(I). Observation with both the model complexes reveal that in the presence of ascorbate, rhodanine and DIDE reacts with Cu(I) to form more stable complexes than the model complexes.

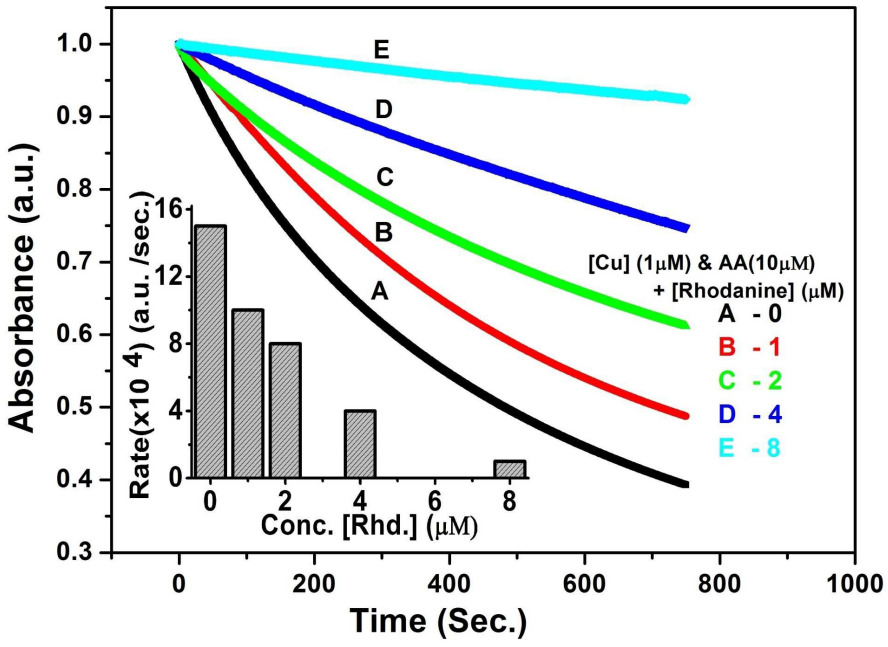


Figure 4: Inhibition of Cu(II) catalysed oxidation of ascorbic acid in presence of varying concentrations of rhodanine (at 265nm, λ_{max} for ascorbic acid). [Cu(II)-1μM, AA-10μM, Rhd.-1-8μM]

The presumption was further validated by monitoring the effect of rhodanine on catalytic behaviour of copper by measuring the rate of copper-enhanced oxidation of ascorbate (Cu(II) itself getting reduced to Cu(I)). Rhodanine (0-8 μM) and Cu(II) (1 μM) were added to a solution of 10μM ascorbate and incubated at physiological conditions (0.1 M phosphate buffer (pH 7.4) and 37^{o}C). The oxidation of ascorbate was monitored spectrophotometrically at 265 nm. The slope of the absorbance versus time curve was observed to decrease with increasing concentration of rhodanine and at rhodanine/Cu ratio of 8, the curve almost flattened which indicates that a remarkable inhibition to the copper catalysed oxidation of ascorbate occurs in presence of rhodanine (Figure 4). Similar observations have been reported by by Zhu et al. [25, 26] for for thiourea and ergothioneine in their studies related to inhibitory potential of these ligands on copper induced oxidative damage to proteins and DNA. These results clearly indicate that addition of the ligands decreases the rate of metal catalysed oxidation of ascorbate. This can be attributed to the oxidation state selective complexation of ligand with Cu(I) [25, 26]. This in turn influences the formation of ROS and hence the rate of ascorbate oxidation.

These observations regarding selective complexation of rhodanine with Cu(I) can be attributed to the fact that rhodanine with a soft donor atom (S) in accordance with the HSAB considerations [33] prefers to bind copper in lower oxidation state. It is not possible to explain the high selectivity of DIDE for Cu(I) on the basis of nature of donor atoms in this biologically active ligand. Structural optimization through computation methods (using DFT theory) of DIDE as free and complexed with Cu(II) and Cu(I), were carried to check the geometrical changes accompanying the complexation of DIDE with Cu(II) and Cu(I) (Figure 5). DIDE and its copper complexes were optimised for the lowest energy geometry using B3LYP functional and LANL2MB basis set.

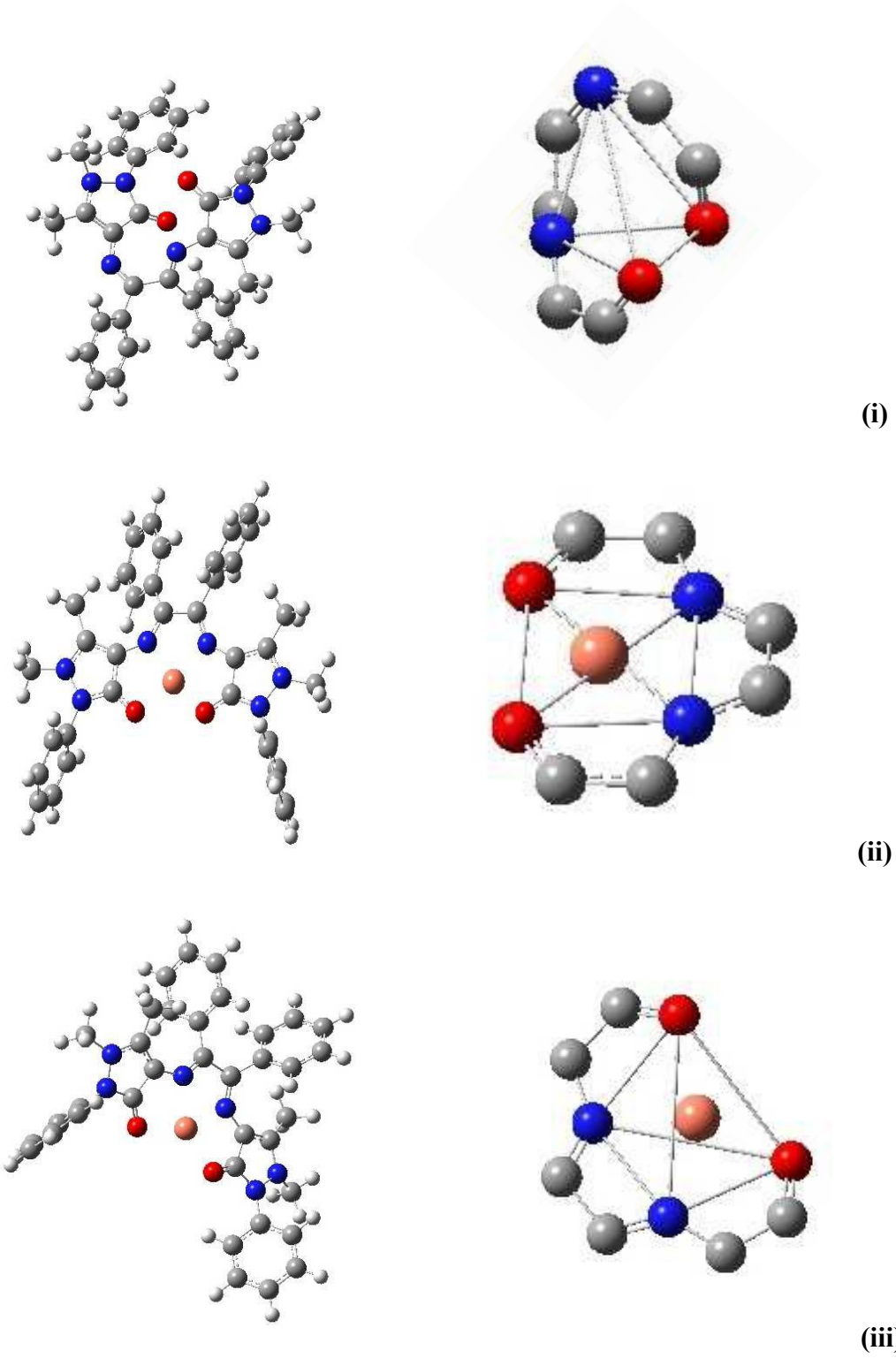


Figure 5: DFT Optimized structures (coordination sphere enlarged) of (i) DIDE, (ii) DIDE-Cu(II) complex, (iii) DIDE-Cu(I) complex.

The polarizable continuum model (PCM) was used for modeling the effect of solvent (water) on the complexation [34,35]. In the lowest energy optimized geometry of DIDE, the four donor atoms were observed to be positioned at the four corners of a regular tetrahedron (Figure 5(i)). On other hand for the formation of most stable geometry of the Cu(II) complex with DIDE (Figure 5(ii)) the four donor atoms of DIDE need to reorient to get positioned at four corners of a plane square. Contrary to this, no reorientation of these four donor atoms is required for the formation of Cu(I)-DIDE complex (Figure 5 (iii)). Positioning of four donor atoms in a tetrahedral geometry for an entropically and enthalpically favoured binding of Cu(I) hence seems to be the prime reason for Cu(I) selectivity of DIDE. It is a known fact that Cu(II) complexes prefer to exist as Jahn–Teller distorted octahedron (though other configurations are known), while Cu(I) complexes usually exist as four-coordinate tetrahedral[28,36]. Complexation with Cu (II) requires DIDE to undergo some structural reorientations against the optimized (lowest energy) structure which makes complexation somewhat unfavourable as compared to Cu(I) wherein the four donor atoms persist with the same spatial arrangement. This preferential affinity for Cu(I) of DIDE hence results in increase in the reduction potential of Cu(II)/Cu(I) couple which is responsible for its observed potential to reduce the copper induced oxidative damage to BSA.

Conclusion:

The present study leads us to hypothesize that the structural/conformational requirements of potential ligands that are capable of inhibiting the Cu –induced oxidative damage should include a soft donor site, donor sites placed at the corners of a tetrahedron and a good π-back acceptance character. Since 1,2-(diimino-4'-antipyrinyl)-1,2 diphenyl ethane (DIDE) and Rhodanine-the two biologically important ligands meet these structural/conformational requirements, they exhibit an excellent anti-oxidant activity by chelation of copper into its redox inactive state. To the best of our knowledge, this study is the first to establish that the antioxidant activity of DIDE and Rhodanine is an outcome of their ability to bind copper into a redox inactive sate. Their selective binding ability for Cu(I) endows them with potential to reduce the generation of ROS. This finding is an important contribution towards the studies for exploring the potential pharmacological potential of these two bioactive molecules. The future studies need to evaluate their protection against free radical-mediated oxidative damage to other types of proteins such as integral membrane proteins that constitute the majority of drug targets, and to nucleic acids such as DNA and RNA. In our next steps we intend to repeat the similar experiments on detergent solubilized and purified Na^+/H^+ transporter protein [37], and later also in-vitro and in-vivo analysis on cellular nucleic acids. The novelty of the present study is that in contrast to the majority of antioxidant studies which are based on free radical scavenging, we have explored a metal-ion complexation based antioxidant screening approach for the search of bioactive ligands with potential to inhibit copper induced oxidative damage to proteins.

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