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Research paper

Structural characterization, ROS-inductive and proteasome inhibitory properties of ternary and binary copper(II) complexes of N_2- and N_2O_2-ligands

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ABSTRACT

The four copper(II) complexes involving ethylenediamine-*N*,*N*-diacetic acid (H_2edda) and ethylenediamine (en), viz. [Cu(edda)(en)]·H_2O **1**, [Cu(en)Cl_2] **2**, [Cu(edda)] **3** and [Cu_2(Hedda)$_2Cl_2$] **4**, were synthesized and characterized by various physical means. The crystal structures of **1** and **4** established them as mononuclear and dichlorido-bridged dicopper(II) complexes respectively. Complexes **1** and **4** showed weak and strong antiferromagnetic Cu. . .Cu interaction. Dynamic light scattering data of **3** suggested it to be a 3-dimensional coordination polymer in aqueous solution, gel and solid forms. The copper(II) species of **1–4** in aqueous solution were analysed by UV–visible and molar conductivity data. The weak hydroxyl radical-inducing property of free copper(II) ions in solution was enhanced by the chelation of both types of ligands. However, chelation of each or both of these ligands reduce the strong proteasome inhibitory property of the copper(II). All complexes inhibited the three proteolytic sites of the 20S proteasome, with the Trypsin-like site been mostly selectively inhibited.

1. Introduction

Copper(II) complexes are widely studied partly due to their rich stereochemistry, and interesting magnetic and redox properties. Ferromagnetic and antiferromagnetic exchange between copper (II) (Cu^{2+}) ions, especially in dimeric copper(II) complexes, are still studied because the role of bridging ligand and hydrogen bonding in intramolecular and intermolecular exchange mechanisms are not fully understood [1,2]. The importance of copper and their complexes can also be seen from their extensive involvement in biological processes in living organisms, diseases, and in various industrial, biological, chemical-bio sensory and therapeutic applications [3–6]. The latter encompasses development of copper complexes against parasites and as anticancer agents [7,8].

One new biological property, which attracts anticancer drug researchers, is inhibition of proteasome which is a new validated target of anticancer compounds [9–12]. The human 26S proteasome is an ubiquitous, multi-catalytic protease which consists of 19S regulatory particles and a 20S proteolytic core [9]. The 20S proteasome has three proteolytic sites, with chymotrypsin-like, trypsin-like and caspase-like activities respectively, capable of degrading different types of peptides or proteins. In comparison to normal cells, cancer cells have been reported to be more sensitive to proteasome inhibition, and the inhibition of chymotrypsin-like activity of the 20S proteasome in cancer cells by copper(II) complexes has resulted in apoptosis of the cells [12–14]. Unlike anticancer drugs like bortezomib, copper(II) complexes are likely to bind to the proteolytic sites by non-covalent interactions without modifying the nucleophilic Thr1 residue [15–17]. The proteasome inhibitory property of a copper(II) complex may be derived from the copper(II) itself or from its coordinated ligand. Copper (II) complex of 2,4-diiodido-6-((pyridine-2-yl-methylamino) methyl)phenol was a potent inhibitor of proteasome activity of live liver cancer cells but its precursors, viz. copper(II) chloride and its free ligand, showed insignificant inhibition [12]. Indole-3-acetic acid and indole-3-propionic acid could not inhibit 20S proteasome but their copper(II) complexes could inhibit 20S and 26S proteasomes [18].

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Among the physico-chemical properties, reactive oxygen species (ROS)-inducing property of copper(II) salts and their complexes has been extensively investigated because of the role of ligands in modulating the ROS-inducing property of the copper (II) when bound to these ligands. Many binary and ternary copper(II) complexes with different types of ligands can cleave DNA oxidatively in the presence of a reductant (e.g. ascorbic acid) or oxidant (e.g. hydrogen peroxide H_2O_2) but the ROS-generating ability of these copper(II) complexes are rarely quantitatively compared by using ROS-detection assay [19–22]. Similarly, there is seldom quantitative comparison of copper(II) complexes with the ROS-generating ability of their precursor copper(II) salts and free ligands. A ligand, N-(2-hydroxyacetonephenone)glycinate (L), did not generate significant amount of ROS in CEM/ADR5000 leukaemia cells but the Cu(L) could [23]. Copper(II) sulphate produces dismutation and reduction of superoxide to peroxide but the binary and ternary copper(II) complexes with salicylic acid and diamine ligands are efficient superoxide DISMUTASE mimetics [24]. The metal-free ligands phen and 2,2′-bipy were low-level ROS generators but the copper(II) complexes incorporating o-phthalate and phen or 2,2′-dipyridyl were significantly better ROS generators [25].

In this paper, we synthesized and characterized the binary and ternary copper(II) complexes of ethylenediamine (en) and ethylenediamine-*N*,*N*′-diacetic acid (H_2edda) before investigating their reaction with H_2O_2 to yield hydroxyl radicals and their ability to inhibit the three proteolytic sites of 20S proteasome. These complexes, viz. [Cu(edda)(en)] **1**, [Cu(en)Cl_2] **2**, [Cu(edda)] **3** and [Cu_2(Hedda)$_2Cl_2$] **4**, have ligands with capability to form H-bonding. The crystal structures of complexes **1** and **4** are also reported herein.

2. Experimental

2.1. Materials and instrumentation

Most of the reagents were of analytical grade and were used without further purification. Hydrogen peroxide stock solutions were freshly prepared, by dilution with ultra-pure water, before use.

2.2. Physical measurements

Shimadzu 8400S FTIR spectrometer was used to obtain the FTIR spectra of the $CuCl_2$ and copper(II) complexes, which were prepared as KBr pellets. UV–visible spectroscopic measurement was carried out on a Perkin–Elmer Lambda 25 spectrophotometer; for studies on aqueous sample solutions, distilled water was filled into the reference cell while for those on buffered solutions, the corresponding buffer was the reference material. Dynamic Light Scattering technique was used to measure particle size in aqueous solution using a Zetasizer NanoZS (Malvern Instruments, UK). A CON 700 bench top conductivity meter from EUTECH Instruments was used to measure the conductivity of the solvents and methanol-water (v/v 1:1) solutions of the copper(II) compounds. The positive-ion electrospray ionization–mass spectra (ESI–MS) of **4**, dissolved in water-methanol (1:1 v/v), was obtained using Thermo Finnigan LCQ mass spectrometer (National University of Singapore) by infusion method with a heated capillary temperature of 60 °C and capillary voltage of −21V.

2.3. Synthesis of copper(II) complexes

Blue crystals of [Cu(edda)(en)]·H_2O **1** were prepared by heating an aqueous solution of electrolyte-free fresh $Cu(OH)_2$ (prepared by $CuCl_2$ (0.2728 g, 1.6 mmol) and NaOH (0.1280 g, 3.2 mmol)), ethylenediamine-*N*,*N*′-diacetic acid (0.2819 g, 1.6 mmol) and ethylenediamine (0.1 ml, 1.6 mmol). On evaporation at room temperature, the resultant solution yielded blue crystals which were washed with cold ethanol and dried overnight in an oven at 55 °C. These crystals were suitable for crystal structure analysis, and crystal structure determination revealed presence of one water molecule. Yield: 44%. Repeated syntheses yielded the complex [Cu(edda)(en)]·1½H_2O with different number of lattice water molecules, as indicated by elemental analysis data. FTIR (KBr) cm^{-1}: 3412 (ν (OH), br), 3250 and 3163 (ν (NH), vs), 2956 (ν (C–H), m), 1597 (ν_{as} (CO), vs), 1392 (ν_s (CO), vs), 1306 (s), 1217 (m), 1086 (s), 1045 (s), 1020 (s), 968 (s), 897 (m), 829 (w), 739 (s), 633 (s), 579 (s), 530 (s), 432 (w). Elemental Analysis: *Anal. Calc.* for [Cu($C_6H_{10}N_2O_4$)($C_2H_8N_2$)]·1½H_2O, i.e. Cu($C_8H_{21}CuN_4O_{5.5}$): C, 29.58%; H, 6.52%; N, 17.25%. Found: C, 29.74%; H, 6.25%; N, 17.35%.

[Cu(en)Cl_2] **2** was prepared by a previously published procedure [26]. Yield: 34%. FTIR (KBr disc) cm^{-1}: 3298.38 and 3228.95 (ν (NH), vs), and 1633.76 (ν (NH), br). Elemental Analysis: *Anal. Calc.* for $C_2H_8CuN_2Cl_2$: C, 12.35%; H, 4.14%; N, 14.40%. Found: C, 12.31%; H, 4.09%; N, 14.36%.

[Cu(edda)] **3** was resynthesized by using the same copper(II) hydroxide method, as reported previously [27]. An excess amount of $Cu(OH)_2$ was first prepared with an aqueous solution of $CuCl_2$ (0.2557 g, 1.5 mmol) and NaOH solution (0.1200 g, 3 mmol). Cu$(OH)_2$ was added into the aqueous solution of ethylenediamine-*N*,*N*′-diacetic acid (H_2edda: 0.1762, 1 mmol) and heated on a hot plate, then in a water bath at 55 °C for 4 h. Evaporation was prevented by heating in sealed vessels. Unreacted $Cu(OH)_2$ precipitate was filtered off with a 0.2 μm syringe filter. Concentration of the aqueous solution of [Cu(edda)] was calculated using the amount of limiting agent, H_2edda. The clear, filtered blue solution was scanned with a Malvern Zetasizer Nano ZS to investigate presence of polymeric species and measure their particle sizes. The dried solid powder of [Cu(edda)] **3** was obtained by repeated treatment of the above aqueous solution with ethanol and acetone to precipitate the solid. Subsequent drying of this solid in an oven yielded the dried blue powder of [Cu(edda)] which was completely dehydrated, as was obtained previously [27].

[Cu_2(Hedda)$_2Cl_2$] **4** was prepared by heating an aqueous solution of $CuCl_2$ (0.8524 g, 5 mmol) with ethylenediamine-*N*,*N*′-diacetic acid (0.8809 g, 5 mmol) on a hot plate, and then in a water bath at 55 °C for 4 h. On standing at room temperature, the solution yielded small lilac prismatic crystals. These were filtered, washed with cold ethanol and dried overnight in an oven at 55 °C. Yield: 14%. FTIR (KBr) cm^{-1}: 3468 (ν (OH), br, w), 3270 and 3196 (ν (NH), s), 2945 (ν (C–H), m), 1697 (ν_{as} (CO of unionized COOH), s), 1547 (ν_{as} (CO of ionized COO^-), vs), 1400 (ν_s (CO), vs), 1465, 1450, 1435, 1275, 1238, 1030, 928 and 702 (edda). Elemental analysis: *Anal. Calc.* for $Cu_2(C_6H_{11}N_2O_4)_2Cl_2$: C, 26.28%; H, 4.04%; N, 10.22%. Found: C, 26.08%; H, 4.00%; N, 10.05%.

2.4. X-ray crystallography for [Cu(edda)(en)]·H_2O **1** and [Cu_2(Hedda)$_2Cl_2$] **4**

Intensity data for a blue plate crystal, 0.40 × 0.08 × 0.03 mm, of [Cu(edda)(en)]·H_2O **1** was collected at −150 °C on a Bruker SMART APEX area-detector and that for a lilac prismatic crystal, 0.50 × 0.35 × 0.25 mm, of [Cu_2(Hedda)$_2Cl_2$] **4** was collected at −173 °C on a Agilent Technologies SuperNova Dual diffractometer with Atlas detector, with both using MoKα radiation (λ = 0.71073 Å). The APEX2 software and SAINT software were used for data acquisition and refinement and data reduction respectively. Both structures were solved by direct-methods and refined by a full-matrix least-squares procedure [28]. The [Cu(edda)(en)]·H_2O **1** complex molecule was found to crystallize with one water molecule. The O-bound H atoms were located from a

difference map and included in the model with O—H constrained to 0.840(1). The molecular structures of **1** and **4** are shown in Figs. 1 and 2 respectively. Their crystallographic data have been deposited at Cambridge Crystallographic Centre (CCDC No. 772109 and 1037882 respectively) which can be obtained from http://www.ccdc.cam.ac.uk/Community/ Requestastructure/Pages/Data Request.aspx.

2.5. PNDA assay for reaction of complex with H_2O_2

To quantify the amount of ·OH radicals produced by the reaction of **1** in borate buffer at pH 7.5, a previously reported *p*-nitrosodimethylaniline (PNDA) assay was used [29,30]. The percentage bleaching of the PNDA was calculated by using the formula, % Bleaching of PNDA = $100 \times (A_o - A_t)/A_o$ where A_o = absorbance of sample with PNDA at 440 nm at t = 0 while A_t = absorbance of sample with PNDA at 440 nm at any time, t.

A total volume of 300 μL of each assay mixture, consisting of 90 μL of test compound (30 μM), with 18 μL of H_2O_2 (60 mM), 141.6 μL of borate buffer (33 mM, pH 7.5) and 50.4 μL of PNDA (42 μM), was placed in a 96-well transparent plate. The absorbance reading at 440 nm was taken immediately after the addition of PNDA by using SpectraMax M5 multi-mode microplate reader and the measurements were run continuously for 4 h with interval of 5 min.

2.6. 20S Proteasome inhibition

Fluorogenic substrates Suc-Leu-Leu-Val-Tyr-AMC, Boc-Leu-Arg-Arg-AMC and Z-Leu-Leu-Glu-AMC (UBPBio USA) were used to measure chymotrypsin-like, trypsin-like and caspase-like activities of the 20S proteasome, respectively. A total volume of 100 μL of each assay mixture, consisting of 14 μL of activated purified 20S mouse proteasome (2 nM/well)(R&D Systems USA), with 20 μL of 20 μM fluorogenic peptide substrate (at 4 μM/well), an appropriate volume of buffer (50 mM Tris-HCl, pH 7.5) and appropriate volume of test compound at indicated concentration (2.5, 5, 10, 20, 30 and 40 μM) in a 96-well fluorometer plate, was incubated for 24 h at 37 °C. After incubation, fluorescence of the cleaved fluorogenic groups was measured by using SpectraMax M5 multi-mode microplate reader with an excitation filter of 380 nm and an emission filter of 460 nm. Activity (%) of each site was calculated as (optical density of sample)/(optical density of control) × 100%. Changes in fluorescence were calculated against non-treated controls and plotted with statistical analysis using Microsoft Excel and the concentration (μM) of samples that induced 50% inhibition of the 20S's three proteolytic sites were determined by using the plot of activity (%) of each site against concentration of test samples.

3. Results and discussion

3.1. Analysis of structures of complexes **1–4**

3.1.1. Crystal structure of [Cu(edda)(en)]·H_2O **1**

The crystal data and selected bond lengths and angles are presented in Supplementary Tables 1 and 2 respectively. The crystal structure of the ternary copper(II) complex of ethylenediamine-*N*,*N*′-diacetate ($edda^{2-}$) and the ethylenediamine (Fig. 1) shows that the $edda^{2-}$ coordinates as a tetradentate (N_2O_2-ligating atoms) while the en coordinates as a bidentate (N,N′-ligating atoms). The crystalline complex has one lattice water molecule, and it can be formulated as [Cu(edda)(en)]·H_2O **1** which has an octahedral geometry about the copper(II) atom. This complex is racemic with the Δ- and Λ-[Cu(edda)(en)] isomers co-existing in the crystal. The number of lattice water molecules ([Cu(edda)(en)]·H_2O; x = 1, 1½) can vary with each synthesis. The tetradentate $edda^{2-}$ coordinates to the copper(II) atom in a similar way as the same ligand in [Cu(phen)(edda)] [31]. Unlike those in the latter {with Cu(1)–O(1) 2.3957(12) and Cu(1)–O(3) 2.3140(11) Å; Cu(1)–N(3) 2.0429(13), Cu(1)–N(4) 2.0263(13) Å}, the two Cu1–O1(carboxylate) and Cu1–O1′(carboxylate) bonds of [Cu(edda)(en)] are equal (with bond length of 2.354(6) Å), and so are the two Cu–N1(amino) and Cu–N1′(amino) bond lengths (2.043(5) Å). In contrast, the two *trans* carboxylate oxygen atoms (O1 and O1′) are coordinated to the Cu1 such that the O1–Cu1–O1′ angle (167.0(3)°) is even more bend than that of [Cu(phen)(edda)] (174.02(4)°). The two Cu1–N(en) and Cu1–N′(en) bond lengths are equal (2.008(6) Å) and the N2-Cu1-N2′ bite angle is 86.2(4)°). Nevertheless, the Cu1 atom still has the usual distorted octahedral geometry.

3.1.2. Structures of [Cu(en)Cl_2] **2** and [Cu(edda)] **3**

The crystal structure of binary copper(II) complex **2** has been reported to be tetrahedral, with a bidentate ethylenediamine and two monodentate chloride ligands coordinated to the copper atom [26]. Complex **3** was synthesized according to a previously published procedure for [M(edda)(H_2O)$_2$] (M(II) = Co, Zn, Cu) [27]. This complex **3** was synthesized and had previously been partially characterized [27]. The synthesis of **3** needs treatment with ethanol, acetone and drying in an oven to recover a dry solid from the aqueous solution; otherwise, a gel or paste or hard transparent solid is recovered upon evaporation of the aqueous solution. Based on FTIR and elemental analysis, it was established that the dried blue powder of **3** was [Cu(edda)]. Unlike the other [M(edda)(H_2O)$_2$] complexes, the crystal structure of this copper(II) complex of ethylene-*N*,*N*′-diacetate, i.e. [Cu(edda)], could not be determined as the dried powder or the gel was always obtained. The gel or harden solid of this complex may be due to its polymeric nature, with the carboxylate COO^- as bridging group. Carboxylate as bridging or linking ligands for metal coordination polymers are

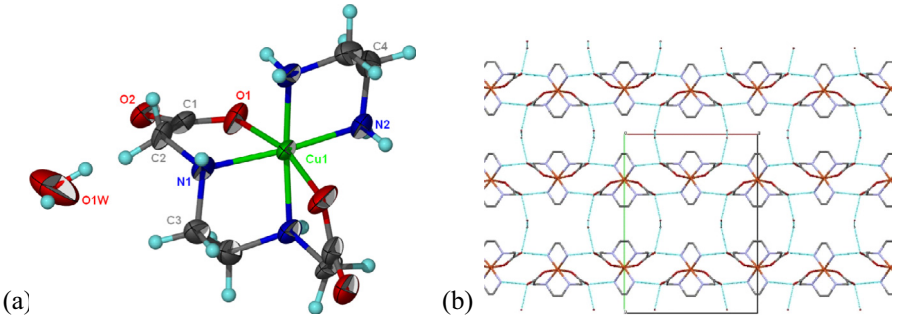

Fig. 1. (a) Thermal Ellipsoid plot of [Cu(edda)(en)]·H_2O 1 is drawn at 50% probability; (b) Hydrogen bonding network (light blue) in **1**, viewed down *c*-axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

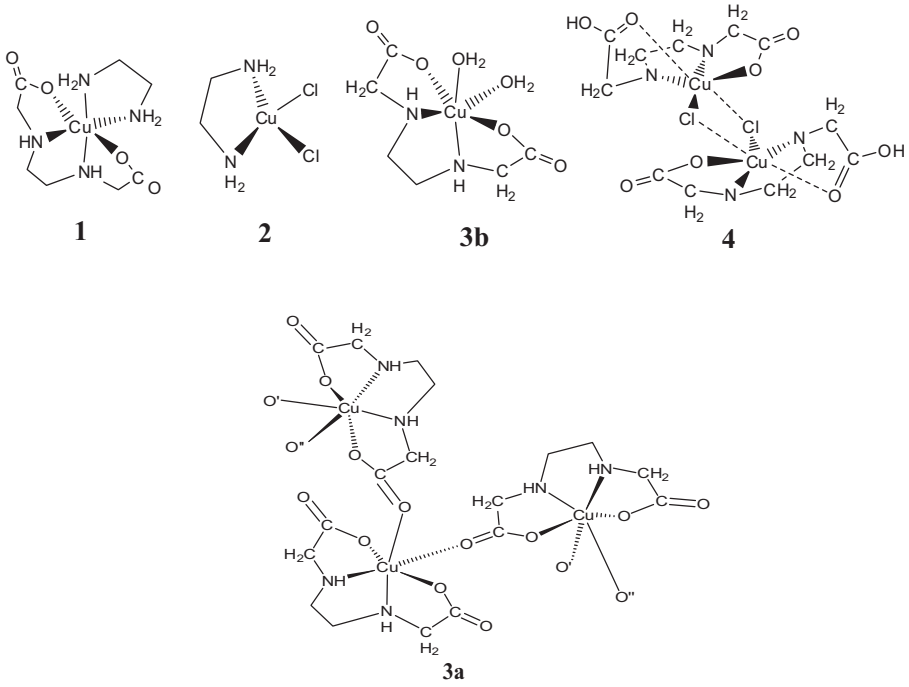

Fig. 2. Structures of [Cu(edda)(en)] (1), [Cu(en)Cl_2] (**2**), [Cu(edda)] (**3**) and [Cu_2(Hedda)$_2Cl_2$] (**4**). *__3b__ = Postulated polymeric [Cu(edda)]$_n$; O' and O" are carbonyl oxygen atoms of different [Cu(edda)] unit.

well known [32–35] The [Cu(edda)] gel is probably a new member of a class of 3-dimensional supramolecules called metallogels, and belongs to the sub-group characterized by chemical metal–ligand coordination interaction in which the interactive strength lies between that of strong covalent bonding and that of other noncovalent interaction [36]. To investigate the particle size of the polymeric species present, dynamic light scattering (DLS) technique was used to study different aqueous solutions of **3** with a Zetasizer. Preliminary particle size measurement results for (i) a freshly prepared aqueous solution of **3** (from $Cu(OH)_2$ and H_2edda, pH 7), (ii) a concentrated solution of **3** which has been obtained by evaporating a freshly prepared aqueous solution to half its original volume (pH 7), and (iii) a diluted solution of some dried powder of **3** (pH 7), show presence of nanosize particles of mean values of 408 ± 13, 790 ± 260 and 146 ± 6 nm respectively (Supplementary Table 3; Supplementary Fig. S1). The viscous gel of **3**, when diluted with water, yields similar nanoparticles (result not given). These results suggest that these different forms of complex **3** are polymeric. Similar presence of nano-sized fragmented species of polymeric copper(II) complex of an amino acid, [Cu(3Me-pic)$_2$]$_n$ (3Me-pic = 3-methyl-picolinate), has been established with the use of a Zetasizer using DLS [37]. Unlike [Cu(3Me-pic)$_2$]$_n$, complex **3** is likely to be a 3-dimensional (3-D) coordination polymer [36,38]. The gel of **3** is then a metallogel, with water molecules trapped in the 3-D polymer network [36].

In the aqueous solution of complex **3**, the individual [Cu(edda)] and [Cu(edda)(H_2O)] species were detected by ESI-MS at 80 °C desolvation temperature whereas an additional [Cu(edda)(H_2O)$_2$] species could surprisingly be detected at higher desolvation temperatures (100 and 150 °C) [27]. The charge transfer band at 249 nm (ε, 3847 M^{-1} cm^{-1}) and the visible d–d transition band centered at 672 nm (ε, 92 M^{-1} cm^{-1}; Table 1) for the aqueous solution of complex **3** was consistent with a six-coordinate copper(II) complexes with distorted octahedral geometry [27]. However, we now think that the species in the aqueous solution of **3** exist as [Cu(edda)]$_n$ species (nano-size fragments of the polymer where the 5th and 6th coordination sites are occupied by bridging carbonyl oxygen atoms of adjacent [Cu(edda)] units). Nevertheless, small amount of octahedral [Cu(edda)(H_2O)$_2$] species may also be present. Under ESI-MS conditions, the polymeric [Cu(edda)]$_n$ **3b** could easily break down to yield [Cu(edda)] and hydrated [Cu(edda)(H_2O)$_2$] **3a** species (Fig. 2).

Table 1
Visible spectral data of the 5 mM of copper(II) complexes.

Aq. Solution	0 h	24 h	48 h
	λ_{max}/nm (ε/mol^{-1} dm^3 cm^{-1})		
$CuCl_2$	818(13)	817(13)	821(14)
[Cu(edda)(en)]·H_2O **1**	628(67)	628(66)	629(68)
[Cu(en)Cl_2] **2**	661(67)	659(66)	661(68)
[Cu(edda)] **3**	672(91)	672(92)	672(92)
[Cu_2(Hedda)$_2Cl_2$] **4**	676(169)	676(166)	676(162)

3.1.3. Crystal structure of [Cu_2(Hedda)$_2Cl_2$] *4*

The crystal data and selected bond lengths and angles are presented in supplementary Tables S1 and S2 respectively. The complex **3** was obtained from the reaction of H_2edda with freshly prepared $Cu(OH)_2$. However, the reaction of H_2edda with only $CuCl_2$ at pH ~ 1.2 yielded a dicopper(II) complex, **4**, which crystallized as lilac crystals. Each dimer molecule consists of two identical subunits or monomers of [Cu(Hedda)Cl], which comprises a coordinated chloride (Cu–Cl, 2.2604(8) Å (s) for the Cu1 unit) and a tridentate, anionic $Hedda^-$(Fig. 3). The coordinated chlorido of each subunit bridges the copper atom of the other subunit *via* a weak interaction, having a Cu1···Cl1 bond of 2.7559(8) Å. The latter [Cu(Hedda)Cl] is similarly bridged to the former to complete the di-μ-chlorido bridged [Cu_2(Hedda)$_2Cl_2$]. The two subunits are thus held together by bridging chlorido ligands. The presence of the carboxylate COO^- and carboxylic acid COOH of each $Hedda^-$ moiety is substantiated by their CO stretching frequencies at 1547 (ν (CO of ionized COO^-), vs) and 1697 (ν (CO of unionized COOH), s) respectively [39].

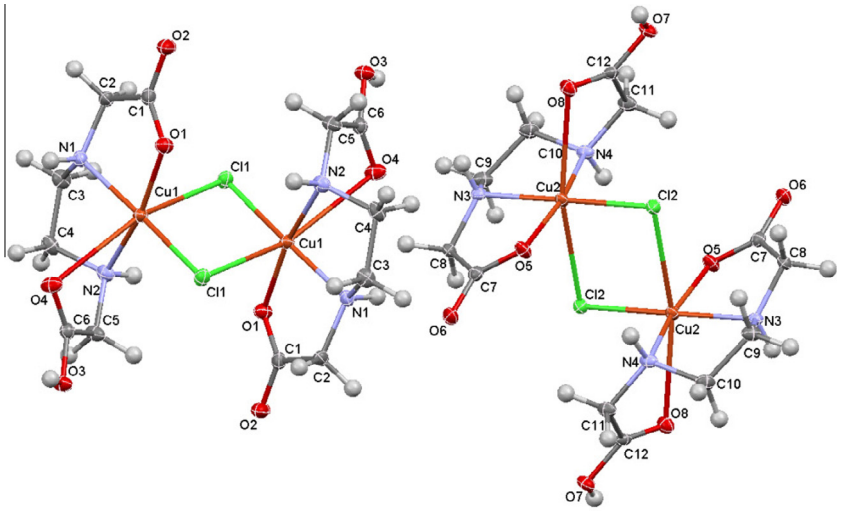

Fig. 3. Ortep plot of the two assymetric $[Cu_2(Hedda)_2Cl_2]$ **4** molecules with ellipsoid at 50% probability.

The geometry about the copper atom in the complex molecule can be considered pseudo-octahedral. The carboxylate oxygen (O1) and two amino atoms (N1, N2) of the tridentate $Hedda^-$ anion, and the coordinated chlorido (Cl1) are distorted from a "basal plane" to form a butterfly-like structure. The carbonyl oxygen atom (O4) of the carboxylic acid group of $Hedda^-$ bends over this butterfly-like structure to interact weakly with the central copper atom (Cu1) (Cu–O(carbonyl), 2.746 Å). The sixth position is occupied by another bridging chlorido atom of the other [Cu(Hedda)Cl] subunit (Cu–(μ-Cl), 2.7559 Å). In each unit cell, there are two asymmetric $[Cu_2(Hedda)_2Cl_2]$ complex molecules, with the same molecular formula $[Cu_2(Hedda)_2Cl_2]$. They differ from each other because their [Cu(Hedda)Cl] subunits are asymmetric. Additionally, the $[Cu_2(Hedda)_2Cl_2]$ molecules are stabilized by intermolecular H-bonding and linked into a 3-dimensional network *via* H-bonds (Fig. 4). The H-acceptor and H-donor involve the carbonyl oxygen atom (H-acceptor) of coordinated carboxylate of $Hedda^-$ moiety, the H-atoms of the carboxylic acid (COOH) and the amine nitrogen the $Hedda^-$ moiety (H-donors).

The two copper and two chlorido atoms in the dicopper molecule form a 4-membered planar ring, in which the Cu1–Cl and Cu1–Cl' distances are 2.2604(8) and 2.7559(8) Å respectively. Such unequal copper-chlorido distances are common in chlorido-bridged copper dimers [40–44]. The Cu···Cu′ distance is 3.4258(6) Å (3.5887(5) Å for other dimer) and this long distance is unlikely to allow direct spin-spin coupling to occur as it is much greater than the interatomic distance of 2.556 Å in metallic copper at 20 °C [45]. The bridging Cu1–Cl1–Cu1′ angle (θ) is 85.54(3)° while the Cl1–Cu1–Cl1′ bite angle is 94.46(3)°. It was found that the Cu(II)-Cu(II) electron exchange coupling constant, 2J, of chlorido-bridged dicopper complexes is related to θ/R where R = longest Cu–(μ-Cl) distance [46]. For $32.6 < (\theta/R) < 35.8$ Å^{-1}, the Cu(II)···Cu(II) interaction is expected to be ferromagnetic while for values of θ/R less than 32.6 Å^{-1} and greater than 35.8 Å^{-1}, the interaction is antiferromagnetic [46]. The θ/R value for $[Cu_2(Hedda)_2Cl_2]$, is 31.0 Å^{-1}, and this indicates antiferromagnetic interaction between the two Cu(II) centers.

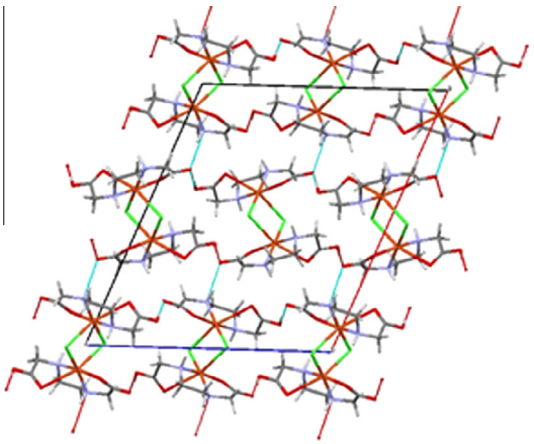

Fig. 4. H-bonding interaction network (blue lines) of $[Cu_2(Hedda)_2Cl_2]$ **4** viewed along the *b*-axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Magnetic property

The experimental values of the magnetic moment (μ_{eff}) of **1**, **2** and **4** are 1.60, 1.75 and 1.27 B.M. at room temperature. The magnetic moment of copper(II) in **2** is close to that of $\mu_s = 1.73$ B.M. with one unpaired electron. The lower magnetic moments of the other two copper(II) complexes suggest varying degree of antiferromagnetic spin-spin coupling interaction. The magnetic moment of each copper(II) in **4** is significantly less than the spin only value, suggesting strong spin-spin coupling (i.e. antiferromagnetic interaction) between the two μ–Cl bridged copper(II) cations [29,47]. The Cu–Cu distance of 3.4258(6) Å (and 3.5887(5) Å for the other dicopper(II) molecule) is too long for direct copper-copper interaction (interatomic distance in metallic copper at 293 K, 2.566 Å). The structural data θ/R for **4** (Section 3.1) also support antiferromagnetic interaction [46]. Similar dicopper complexes, with Cu–Cu distance of between ~3.6 and 7.6 Å and various types of bridging moieties, have been shown unambiguously to exhibit spin-spin interactions *via* a superexchange mechanism rather than a direct copper-copper interaction [48]. The type of interaction between the two copper ions greatly depends on the type of bridging groups. Interestingly, the di(μ-chlorido)-bridged dicopper complexes, $[Cu_2L_2(\mu\text{-}Cl)_2][ClO_4]_2$ and $[Cu_2L_2(\mu\text{-}Cl)_2]Cl_2{\cdot}2H_2O$ (with μ_{eff} values of 1.89 and 1.95 B.M respectively; L = 1,4,7-trimethyl-1,4,7-triazacyclononane), exhibit significantly higher magnetic moment per Cu than that of the present $[Cu_2(Hedda)_2Cl_2]$ **4** (μ_{eff} values of 1.27 B.M.) [48]. Five antiferromagnetic dicopper(II) complexes with doubly-bridged methoxo, hydroxo or chlorido groups have also been shown to have higher magnetic moment

(1.55–1.67 B.M.) at room temperature [47]. Thus, involvement of other spin-spin coupling mechanism or other factors (e.g. hydrogen bonding and magnetic interaction among metal centers) cannot be discounted. In contrast, a di-chlorido-bridged dimeric copper(II) complex, $[L^{Aph}CuCl]_2$, with a tridentate, pyridine based aminophenol ligand (L^{Aph}) shows no antiferromagnetic exchange interaction, and this was ascribed to structural distortion in the square pyramidal geometry around the copper(II) centers [49].

Unlike $[Cu_2(Hedda)_2Cl_2]$, the $[Cu(edda)(en)]\cdot H_2O$ complex does not have any bridging moiety to conduct the antiferromagnetic interaction between copper(II) centers. However, the hydrogen bonding network involving the lattice water molecule, ethylenediamine and ethylenediamine-N,N′-diacetate (Fig. 1b) may provide a weak antiferromagnetic interaction pathway among the copper(II) centers. When the $[Cu(edda)(en)]\cdot H_2O$ is dehydrated, the anhydrous [Cu(edda)(en)] was found to have a magnetic moment of 1.78 B.M. which is the value expected of an unpaired electron in monocopper(II) complexes. This clearly supports the role of H-bonding, due to the presence of lattice water molecules, towards the reduction in magnetic moment of the copper(II). In fact, such involvement of intermolecular hydrogen bonding to enable magnetic exchange interactions of metal centers has only recently been discovered and established [49]. Hitherto, magnetic exchange interactions have acted *via* direct and superexchange mechanisms between metal centers or metal centers and various ligands [50].

Additionally, the strength of the magnetic interaction of the two Cu d-orbitals (containing unpaired e) in the dicopper complexes can spatially be moderated by μ-bridging ligand and/or H-bond network. Copper(II) coordination geometry (including distortion) may moderate d-p orbital interaction to increase or decrease the energy gap between the two molecular orbitals (φ_a and φ_s) of the dicopper(II) interacting d-orbitals so as to change the degree of e-pairing (i.e. the strength of the antiferromagnetic interaction) according to Hoffmann's rule [51]. It is tempting to postulate that the bridging ligand is a better "superexchange conductor" than H-bond network in magnetic interaction of dicopper(II) complexes.

3.3. Solution property

UV–visible spectroscopy and conductivity measurement were used to characterize the copper(II) salts and the four copper(II) complexes in water-methanol (1:1 v/v) solutions (at 4 mM) and to monitor their stability in aqueous solutions by recording their absorbance and λ_{max} at 0, 24 and 48 h. These two methods are simple to use and are regularly utilised [52]. The aqueous copper(II) chloride, containing hydrated copper(II) ions, has λ_{max} at 818 nm due to d-d transition. The λ_{max} values of the aqueous solutions of $[Cu(edda)(en)]\cdot H_2O$ **1** (627 nm), [Cu(edda)] **2** (671 nm), $[Cu(en)Cl_2]$ **3** (660 nm) and $[Cu_2(Hedda)_2Cl_2]$ **4** (675 nm) are shifted to shorter wavelengths, establishing that the ligating ligands with 2 or 3 ligating N-atoms, viz. $edda^{2-}$ (N_2O_2-atoms), en (N_2-atoms), $Hedda^-$ (N_2O-atoms), are stronger field ligands than H_2O. The λ_{max} of aqueous solution of [Cu(edda)] is only slightly lower than that of $[Cu_2(Hedda)_2Cl_2]$, suggesting the same or very similar coordination environment of ligating atoms. The dicopper(II) molecule may have dissociated into two $[Cu(Hedda)(H_2O)_2]^+$ and two Cl^- ions. The aqueous solutions of [Cu(edda)], $[Cu(en)Cl_2]$ and $[Cu_2(Hedda)_2Cl_2]$ have very similar λ_{max} and are comparable to other complexes such as bis(aminoacidato)copper(II) complexes, which have two nitrogen atoms coordinated to copper [53,54]. The [Cu(edda)(en)] has much lower λ_{max} because there are four nitrogen atoms coordinated to its copper atom and N-type ligands have comparatively higher ligand field strength. Many other copper(II) complexes with ligands containing a total of four ligating N-atoms have similar low λ_{max} values (545–640 nm) [55].

The broad, relatively weak bands of the aqueous solutions of the copper(II) complexes, **1** and **2** (Table 1) are characteristic of d-d transitions known for hexacoordinated copper(II) complexes with distorted octahedral geometry and where parity-forbidden Laporte selection rules operate to disallow intense d-d transition [55,56]. Both complexes **3** and **4** have significantly higher molar absorptivities (about 90–170 $mol^{-1}\ dm^3\ cm^{-1}$), suggesting strong tetragonal distortion of the octahedron or square pyramidal geometry where parity-forbidden Laporte selection rules are relaxed by lowered symmetry [57].

The λ_{max} values of the aqueous solutions of the above copper(II) complexes **1–4** did not change appreciably over time, suggesting no change in the coordination environment about the copper atom, and no dissociation of the stronger field ligands (i.e. $edda^{2-}$, en, $Hedda^-$) (i.e. aquation). Thus, they are stable over at least 48 h (Table 1). Additionally, the visible spectrum of $[Cu_2(Hedda)_2Cl_2]$ dissolved in DMSO has λ_{max} values of 690–692 nm within 48 h, suggesting their stability in DMSO solution.

Conductivity measurements for $CuCl_2$ and the complexes **1–4** were performed at 0, 24 and 48 h. Mean values of the molar conductivity of the compounds are given in Table 2. The molar conductivity of **1** in aqueous solution is very low, and is typical of non-electrolytes. Thus, the neutral molecules of this complex are also found in the aqueous solution. Over 48 h, its molar conductivity remained very low, suggesting no dissociation of ligands. The molar conductivity of **2**, $Cu(NO_3)_2{\cdot}2H_2O$ and $CuCl_2$ are about the same, and they show values similar to other 2:1 electrolytes [52,58]. This evidence suggests that each molecule of **2** has dissociated into cationic $[Cu(en)]^{2+}$ (which then formed its hydrated species, $[Cu(en)(H_2O)_4]^{2+}$ and $2Cl^-$ ions upon dissolution in water-methanol). Similar to the stability of [Cu(edda)(en)] species in solution, the $[Cu(en)(H_2O)_4]^{2+}$ species seems to be stable over 48 h as there was no change in molar conductivity. The aqueous solution of **3** has low molar conductivity value (about 16 $S\ cm^2\ mol^{-1}$) which remained unchanged over 48 h. This value is still within the range of other neutral copper(II) complexes [52]. The molar conductivity of $[Cu_2(Hedda)_2Cl_2]$ **4** was found to be about 260 $S\ cm^2\ mol^{-1}$ which is about two fold higher than those of the 2:1 electrolytes (viz. $Cu(NO_3)_2$, $CuCl_2$, $Cu(en)Cl_2$). Each **4** is postulated to have dissociated into two $[Cu(Hedda)]^+$ (exist as $[Cu(Hedda)(H_2O)_2]^+$) cations and two chloride ions when dissolved in aqueous solution. The μ-chlorido bridge in similar dicopper(II) complexes have been found to easily dissociate in aqueous solution [59,60]. Analysis of ESI-MS data of a methanolic solution of **4** confirmed the presence of $[Cu(Hedda)]^+$ which was detected as a molecule ion peak (100%) (Supplementary Fig. S2 and Table 4). The higher number of ionic species per $[Cu_2(Hedda)_2Cl_2]$ can account for the higher molar conductivity of its aqueous solution. However, $[Cu_2(Hedda)_2Cl_2]$ dissolved in DMSO (5 mM) has a mean

Table 2
Molar conductivity of 1 mM of water-methanol (1:1 v/v) solutions of copper(II) salts and copper(II) complexes at room temperature.

Compounds	Molar conductivity, Λ_m ($S\ cm^2\ mol^{-1}$)			Postulated species in aqueous solution
	0 h	24 h	48 h	
$Cu(NO_3)_2{\cdot}3H_2O$	112 (±3)	111 (±2)	112(±2)	$[Cu(H_2O)_6]^{2+}$, $2NO_3^-$
$CuCl_2{\cdot}2H_2O$	123(±2)	124(±3)	124(±3)	$[Cu(H_2O)_6]^{2+}$, $2Cl^-$
[Cu(edda)(en)]·H_2O **1**	8.9 (±0.5)	12.1 (±0.5)	14.0 (±0.5)	[Cu(edda)(en)]
$[Cu(en)Cl_2]$ **2**	119(±3)	119(±3)	119(±4)	$[Cu(en)(H_2O)_4]^{2+}$, $2Cl^-$
[Cu(edda)] **3**	16.4 (±0.3)	16.3 (±0.1)	16.3 (±0.1)	$[Cu(edda)(H_2O)_2]_n$
$[Cu_2(Hedda)_2Cl_2]$ **4**	261(±2)	247 (±10)	230(±9)	$2[Cu(Hedda)(H_2O)_2]^+$, $2Cl^-$

molar conductivity of about 14 S cm^2 mol^{-1}, indicating the dimer molecule is a non-electrolyte in DMSO and did not dissociate. The molar conductivity of this DMSO solution remained practically unchanged (11–14 S cm^2 mol^{-1}) over 48 h, suggesting no dissociation of the neutral dimeric species.

3.4. ROS-generating property

The study of the reaction of inorganic copper(II) complexes and copper(II) enzymes or copper(II) bound to peptides or amino acids with hydrogen peroxide are gaining in importance because hydrogen peroxide is found in all cell types in the human body and different tissues are exposed to different level of endogenous hydrogen peroxide (H_2O_2) [61]. To compare the ability of the copper(II) complexes and the $CuCl_2$ to produce ROS, their reaction with H_2O_2 in borate buffer at pH 7.5 was chosen. Copper(II) complexes can react with H_2O_2 to generate hydroxyl radicals (·OH), whose formation has been successfully monitored by p-nitrosodimethylaniline (PNDA), which reacts quantitatively with the latter at a 1:1 mol ratio (PNDA: ·OH) [27,28]. The decrease of PNDA absorbance at 440 nm is directly proportional to the production of ·OH. In the present experiment, 30 μM of each copper(II) compound was reacted with excess of H_2O_2 (60 mM) in the presence of PNDA (42 μM), and the absorbance of PNDA at 440 nm was measured at fixed interval over a period of 4 h. The absorbance of PNDA against time for these reactions were plotted (Fig. 5) and a comparison of the% bleaching of PNDA for the copper(II) compounds are also given (Table 3).

As can be seen from Fig. 5, the absorbance of PNDA for the $CuCl_2$ reaction decreased slowly with time, suggesting slow production of hydroxyl radicals. Within the first 100 min, the rate of decrease of PNDA absorbance was the fastest for [Cu(en)Cl_2] **2**, followed by [Cu_2(Hedda)$_2Cl_2$] **4**, [Cu(edda)] **3**, [Cu(edda)(en)] **1** and $CuCl_2$, suggesting the compounds can be arranged in same order of decreasing rate of production of ·OH, i.e. from [Cu(en)Cl_2] to $CuCl_2$. The rates of production of ·OH radicals by **4** and **3** are comparable. The rate of the reaction of [Cu(edda)(en)] **1** with H_2O_2 was initially slow within the first 100 min but increased rapidly from 100 to 150 min. The total bleaching of PNDA by all the four copper(II) complexes was practically the same at the end of 240 min (Table 3), and this may signify the end of the reaction or the reaction had become too slow to be monitored. In contrast, the total production of ·OH produced by $CuCl_2$ was less than one fifth. This suggests that chelation of en, $edda^{2-}$ or $Hedda^-$ to copper(II) have significantly enhanced the rate of production of ·OH. The order of copper(II) compounds as increasing generator of ·OH is $CuCl_2$ < **1** < **3** ≃ **4** < **2**. Other ligands are known to affect the ·OH production by copper (II) of these ligands [17]. The ligands in these copper(II) complexes may have lowered the redox potential of the copper centre, resulting in the ease for a Fenton-like Cu^{2+}/Cu^+ cycling in their reaction with H_2O_2 acting as both oxidant and reductant to yield ·OH radicals. The reaction mechanism may involve the following equations which have been proposed previously [29,62,63].

$$Cu^{2+}\text{-L} + H_2O_2 \rightarrow Cu^{+}\text{-L} + O_2^{-\cdot} + 2H^+ \quad (1)$$

$$Cu^{+}\text{-L} + H_2O_2 \rightarrow Cu^{2+}\text{-L} + {\cdot}OH + OH^- \quad (2)$$

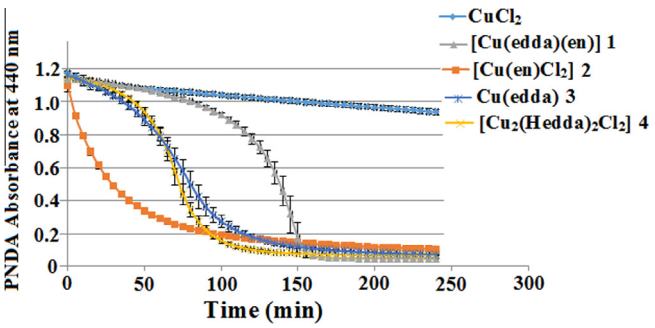

Fig. 5. Plot of absorbance of PNDA at 440 nm against time.

3.5. 20S Proteasome inhibition

Proteasome is responsible for the degradation of 80% of cellular proteins, and its dysregulation is responsible for pathogenesis of many diseases, including Parkinson's disease and cancer [63]. The 26S proteasome is a multiunit proteolytic complex consisting of a 20S cylindrical core and one or two 19S regulatory caps [8,65]. Proteasome inhibitors have potential to treat a wide variety of diseases, including T.B. bacterial infection, cancer and against parasitic protozoa [7,66–68]. The 20S proteasome has three types of proteolytic sites, viz. β5 [Chymotrypsin-like (CT-L)], β2 [Trypsin-like (T-L)] and β1 [Caspase-like (C-L)]. Although numerous copper(II) complexes are known to inhibit proteasome, their "proteasome inhibition profile", in terms of these three proteolytic sites, have not been investigated, as far as we know [69,18]. As part of our programme of characterizing the proteasome inhibition profile of copper(II) and other metal complexes, we herein report the inhibition of the three proteolytic sites of 20S mouse proteasome by the selected series of copper(II) complexes and $CuCl_2$.

The concentration at which a given compound inhibits 50% of a given biological property, i.e. IC50, is normally used to compare the potency of different compounds. Epoxomicin is a very potent covalent proteasome inhibitor and has been used as a positive control. The IC50 values of $CuCl_2$ and the copper(II) complexes were determined (Table 4 and Fig. 6). At about 0.05 μM, epoxomicin inhibits more than 95%, 50% and 40% of the CT-L, T-L and C-L activities of the 20S mouse proteasome. The strong inhibition of epoxomicin may be attributed to its covalent interaction with the nucleolytic threonine moiety at the three proteolytic sites of the proteasome. In terms of inhibition of the proteolytic sites, the profiles of the copper(II) compounds tested are different. Among the copper(II) complexes, **1** is the weakest inhibitor with IC50 values of more than 40 μM for all three sites. All the copper(II) compounds investigated show the least inhibition of the CT-like activity, whose inhibition can lead to apoptosis [12,13,18]. Interestingly, **3** is the most selective inhibitor for the T-L activity as its IC50 is about one-fifth of that of CT-like and one-third that of the C-L activities respectively. Such selectivity for T-L activity of a proteasome inhibitor was found to be useful in sensitizing myeloma cancer cells to bortezomib and carfilzomib [70]. In fact, the cytotoxicity of proteasome inhibitors did not correlate with inhibition of chymotrypsin-like site and that co-inhibition of either trypsin-like and/or caspase-like sites was needed to achieve maximal cytotoxicity [64,71,72].

The precursor $CuCl_2$, used to synthesize the copper(II) complexes (**1–4**), is quite a potent proteasome inhibitor and has lower IC50 values for all three sites than those of the latter compounds. This suggests that the ligands of these copper(II) complexes impart a negative chelation effect. From the IC50 values of **2** and **3**, it can be concluded that **2** is a better proteasome inhibitor than **3** for all three sites by a factor of about 3–7 times. This may be partly due to **2** existing as cationic $[Cu(en)(H_2O)_4]^{2+}$ species which bound more strongly, *via* electrostatic forces, at the proteolytic sites. Similar explanation can account for the strong proteasome inhibition of $CuCl_2$ at all three proteolytic sites as it exists as cationic, hydrated Cu(II) ions.

Table 3
Production of ·OH radicals as measured by bleaching of PNDA.[a]

Complexes	% of PNDA bleaching at different time (min)					
	40 min	80 min	120 min	160 min	200 min	240 min
$CuCl_2$	5.9	8.1	10.3	12.4	14.3	16.3
[Cu(edda)(en)]·H_2O **1**	3.0	10.5	29.7	91.8	92.6	92.2
[Cu(en)Cl_2] **2**	62.5	77.4	82.5	84.7	86.1	86.8
[Cu(edda)] **3**	15.1	55.4	82.4	88.0	89.6	90.1
[Cu_2(Hedda)$_2$$(Cl)_2$] **4**	10.0	68.6	89.1	90.8	90.9	90.6

[a] [·OH] is directly proportional to the % bleaching of the PNDA which is bleached quantitatively by ·OH in a 1:1 ratio (PNDA:·OH).

Table 4
IC50 of epoxomicin, $CuCl_2$ and the copper(II) complexes.

Compounds	Concentration (μM) of the inhibitors that induced 50% inhibition of 20S mouse proteasome activities		
	Trypsin-like	Chymotrypsin-like	Caspase-like
Epoxomicin	0.050	0.005	0.162
[Cu(edda)(en)]·H_2O **1**	>40	>40	>40
[Cu(en)Cl_2] **2**	2.2	9	4
[Cu(edda)] **3**	6.4	35	21
[Cu_2(Hedda)$_2$$(Cl)_2$] **4**	15.6	>40	17.8
$CuCl_2$·$2H_2O$	1.6	8.2	3.2
H_2edda	>40	>40	>40
en	>40	>40	>40

The difference in proteasome inhibition between **2** and **4** (postulated to dissociate to yield [Cu(Hedda)$(H_2O)_2$]$^+$ species) may be due the difference in the charge of their cationic species in solution. In contrast, complex **3** exists as neutral molecule and as such could only bind weakly by hydrogen bonding. By comparing the IC50 values of **2** and **3** with that of $CuCl_2$, it seems that neutral en ligand (N_2-ligand) exhibited minimal negative chelation effect on the proteasome inhibitory property of copper(II) while dianionic edda^{2-} (N_2O_2-ligand) diminished more greatly the inhibition of the T-L, CT-L and C-L activities by 4, 4 and 7 times respectively. The negative effect of en and edda^{2-} is highest for the CT-L site. The mechanism of inhibition by the copper(II) complexes is postulated to be due to their binding at the proteolytic sites *via* electrostatic forces or hydrogen bonding but its validation is currently been investigated. In contrast, it is now known that the mechanism of proteasome inhibition by organic compounds is either of two kinds, viz. by covalent binding with the threonine nucleophile or by non-covalent binding at the proteolytic sites. Examples of the former include epoxomicin, bortezomib and carfilzomib [70] while examples of the latter are TMC-95 and various amides [73].

4. Conclusion

Among the complexes [Cu(edda)(en)]·xH_2O **1**, [Cu(en)Cl_2] **2**, [Cu(edda)] **3** and [Cu_2(Hedda)$_2Cl_2$] **4** complexes, **1** and **3** yielded neutral copper(II) species in aqueous solution whereas **2** and **4** dissociated to form cationic copper(II) species by releasing their chloride ligands. Complex **3** could exists as [Cu(edda)$(H_2O)_2$] and [Cu(edda)]$_n$ species. Both neutral and cationic copper(II) species seem to be stable within 48 h, and the ROS-generating and proteasome inhibition properties can be attributed to these species. The ligand chelation effect of the en, Hedda$^-$ and edda^{2-} ligands on the properties of copper(II) can be positive (enhancement) or negative (decrement). The neutral complex **1** in aqueous solution appears to be the least active with regards to these two properties, both of which could be explained by absence of vacant coordination site at copper(II) for H_2O_2 binding and by weaker binding of **1** to the proteolytic sites respectively. It seems that the proteasome inhibition of cationic copper(II) complex species is generally greater than that of neutral copper(II) species, implying the greater role of electrostatic attraction over other non-covalent binding factors. Nevertheless, all four complexes can inhibit the three proteolytic sites of the 20S proteasome, and they are more selective towards the T-L site. Complexes **2** and **3** are potent inhibitors of the T-L activity.

The solid state mononuclear **1** has a magnetic moment of 1.6 B. M. at room temperature and its lower value than that of the spin only value (μ_s) suggests weak antiferromagnetic interaction, which is postulated to occur *via* the H-bonding network. However, the di-μ-chlorido bridged **4** has an unusually stronger antiferromagnetic

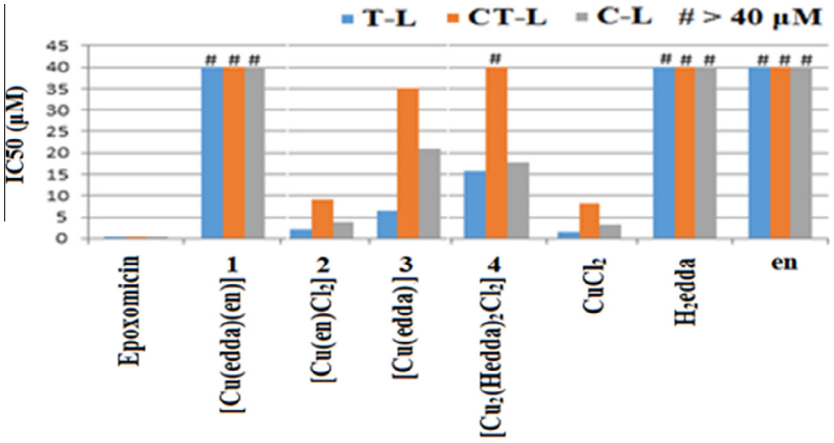

Fig. 6. Bar plots of the IC50 values of epoxomicin, $CuCl_2$ and the copper(II) complexes towards the trypsin-like (T-L), chymotrypsin-like (CT-L) and caspase-like (C-L) proteolytic activities.

interaction (between their copper(II) centers) than other similar di-μ-chlorido bridged dicopper(II) complexes.

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Appendix A. Supplementary data

Table S1 Crystal structure details of [Cu(edda)(en)]·H_2O **1** and [Cu_2(Hedda)$_2Cl_2$] **4**; **Table S2** Selected bond lengths (Å) and angles (°) of complexes **1** and **4**; **Table S3** Mean size (nm) and polydispersity index (PdI) of various aqueous solutions of [Cu(edda)] **3**; **Fig. S1** ESI-MS spectrum of [Cu_2(Hedda)$_2Cl_2$] **4** in water-methanol (1:1, v/v) obtained by infusion method with capillary temperature of 60 °C; **Table S4** Assignment of m/z peaks. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ica.2016.06.003.

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