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Synthesis, characterization and potent superoxide dismutase like activity of novel bis(pyrazole) – 2,2'-bipyridyl mixed ligand copper (II) complexes

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Abstract

Eleven new complexes of Cu(II) chloride and nitrate with bis(pyrazol-1-yl)propane and bis[2-(pyrazol-1-yl)ethyl]ether ligands were prepared and characterized by spectral and electrochemical methods. X-ray crystal structure determination of bis[2-(3,5-dimethylpyrazol-1-yl)ethyl] etherdinitratocopper revealed a hepta-coordinated structure with the bis(pyrazole) ligand coordinated in a tridentate NNO-fashion and both of the nitrate ions in a bidentate fashion. Reaction of Cu(II) nitrate complexes with 2,2'-bipyridyl led to the displacement of one of the nitrate ions into the outer sphere and the formation of mixed-ligand complexes. Mixed-ligand bipyridyl Cu(II) complexes demonstrated the highest superoxide dismutase (SOD)-like activity in a chemical superoxide anion-generating system, with IC₅₀ values in the low micromolar range. Density functional theory calculations showed that introduction of a bipyridyl ligand into the complexes dramatically lowered the lowest unoccupied molecular orbital (LUMO) energy level, which explains the increased SOD-like activity of these complexes compared to non-bipy species. These bipy complexes were also effective scavengers of reactive oxygen species generated by phagocytes (human neutrophils and murine bone marrow leukocytes) *ex vivo*. Thus, these bipy mixed-ligand complexes represent a promising class of SOD mimetics for future development.

Keywords

bis(pyrazole) ligands; copper complex; mixed ligand complex; bipyridyl; superoxide dismutase mimetic

Introduction

Reactive oxygen species (ROS) are produced during cellular processes and drug metabolism. For example, the electron transport chains of the NAD(P)H oxidase, mitochondria, and microsomal cytochrome P450 enzymes are primary sources of ROS.^{1–3} However, excessive ROS formation may lead to oxidative stress and tissue injury, including DNA strand breakage, lipid peroxidation, and other molecular damage. Indeed free radical-induced damage plays an

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Supplementary information

Supplementary information containing the atomic coordinates for all of the DFT optimized structures is available through the online version of this article.

important role in radiation injury and the pathogenesis of many inflammatory diseases, such as atherosclerosis and cancer.^{4,5} Superoxide dismutase (SOD) is one of the most important antioxidant enzymes due to its ability to protect cells from ROS damage by catalyzing the dismutation of superoxide anion radical ($O_2^{\bullet-}$) to O_2 and H_2O_2 .⁶

Although Cu_2Zn_2SOD has been proposed for clinical uses,⁷ this enzyme has several limitations as a pharmacological agent, including high cost, low lipid solubility, low penetration into cells, immunogenicity, and lability to gastric and intestinal proteases.⁸ To overcome these limitations, stable, non-toxic low molecular weight complexes of Cu(II) can be used as SOD mimetics and have potential as pharmacological agents and radioprotectants.

While many small-molecule SOD-mimetic Cu(II) complexes have been reported (e.g.,⁹⁻¹¹), only a few are both thermodynamically stable and highly active in the range of physiological pH.¹² In previous studies, we reported novel Cu(II) bis(1-pyrazolyl)methane complexes with a broad range of antioxidant activity.¹³ The coordination properties of pyrazole ligands can be altered widely by introducing various substituents into the pyrazole rings and additional co-ligands.¹⁴ Thus, these properties may be exploited in the development of useful SOD mimetics. Varying co-ligands can create some interesting variations in the coordination sphere and electronic structure of Cu(II) complexes, which could lead to changes in SOD-like activity and other biochemical behaviors. Previously, 2,2'-bipyridyl (bipy) was intensely used as a co-ligand for modification of metallo-complexes with DNA-intercalative properties.^{15,16} However, the biological properties of mixed-ligand bis(1-pyrazolyl)alkane-Cu(II) complexes have not been studied thus far.

In the present report, we describe the synthesis of novel Cu(II) complexes with bis(pyrazole) ligands, as well as their mixed-ligand bipy derivatives. Spectroscopic, electrochemical, and biochemical properties of these complexes are presented, including evaluation of SOD-like activity in chemical and cellular systems and density functional theory (DFT) study of their electronic structure.

Experimental

Materials

Commercial reagents and solvents were used as obtained without further purification. 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4(2*H*,3*H*)-dione (L-012) was purchased from Wako Chemicals (Richmond, VA). Bovine serum albumin (fraction V) was purchased from Roche Diagnostic (Indianapolis, IN). SOD from horseradish, phorbol-12-myristate-13-acetate (PMA), zymosan A from *Saccharomyces cerevisiae*, phenazine methosulfate (PMS), NADH, and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, MO).

Physical measurements

Conductivities of solutions ($c=1.0 \cdot 10^{-3}$ mol/L in acetone) were measured in a temperature-controlled cell with stainless steel electrodes; cell constant $\phi=0.251$ cm⁻¹. Elemental analyses were carried out on a Carlo Erba analyzer. Infrared (IR)-spectra of solid samples as KBr pellets were recorded on a Nicolet 5700 (4000-400 cm⁻¹) spectrophotometer. Electronic absorption spectra of samples dissolved in either ethanol or 0.05 M phosphate buffer (pH 7.5) were registered on Perkin Elmer 124 (in the range of 200-800 nm) and SF-26 (800-1200 nm) spectrophotometers. NMR spectra were recorded on a Bruker AV300 instrument.

Cyclic voltammograms were obtained using a TA-4 voltammetric analyzer (Tomanalyt, Tomsk, Russia). The three-electrode electrochemical cell consisted of a mercury-film working electrode and saturated Ag/AgCl reference and auxiliary electrodes. Concentration of the

sample in the cell was 10^{-4} mol/L, while 0.1 mol/L KCl was used as a supporting electrolyte. Potential was varied in the range of 0 to -1.3 V at 50 mV/s scan rate. Oxygen was removed from the solutions by bubbling N_2 immediately before measurements. Formal potentials were calculated as $E_{1/2} = (E_{p.c.} + E_{p.a.})/2$, $\Delta E = E_{p.a.} - E_{p.c.}$.

Crystal structure determination of complex 4

Single crystals of complex **4** were mounted in inert oil and transferred to the cold gas stream of the diffractometer. The structure of the complex was determined at 153 K by conventional single crystal X-ray diffraction techniques using an automated four-circle Rigaku RAXIS-spider diffractometer equipped with a 2-D CCD detector and graphite monochromated molybdenum source ($\lambda = 0.71073$ Å). Intensity data were collected by ϕ -scanning of narrow frames (0.5°) to $2\theta = 54.96^\circ$. Empirical absorption correction was applied empirically by the program SADABS.¹⁷ The structure was solved by the direct method and refined using the full-matrix least-squares technique in the anisotropic approximation for non-hydrogen atoms with the program package SHELX-97.¹⁸ Hydrogen atoms were included in geometrically calculated positions.

Crystal data— $C_{14}H_{22}CuN_6O_7$, $M = 449.92$, monoclinic, $a = 17.4261(7)$, $b = 10.3459(3)$, $c = 11.1392(3)$ Å, $\beta = 107.1200(10)$ deg. $U = 1919.29(11)$ Å³, $T = 153(2)$ K, space group $C2/c$ ($No. 15$), $Z = 4$, calc. density 1.557 Mg/m³, 9186 reflections measured, 2202 unique ($R_{int} = 0.0175$), which were used in all calculations. $R1 = 0.0222$ (refined data) and 0.0239 (all data). The final $wR2$ factors were 0.0594 (refined data) and 0.0602 (all data). Goodness-of-fit was 1.149 ; maximum and minimum electron densities were 0.317 and -0.261 , respectively.

Synthesis of ligands and complexes

1,3-Bis(pyrazol-1-yl)propane (L1, L2) and bis[2-(pyrazol-1-yl)ethyl]ether (L3, L4) ligands were prepared as previously reported.¹⁹ Cu(II) complexes **12–18** were prepared using previously reported methods.²⁰

1,4-Bis[bis(3,5-dimethylpyrazol-1-yl)methyl]benzene—(L5) was prepared as described.²¹ Briefly, 3,5-dimethylpyrazole (2 g, 20.8 mmol), finely powdered potassium hydroxide (2.33 g, 41.6 mmol), and 10 mL DMSO were stirred at 80°C for 2 hr, followed by drop-wise addition of 1,4-bis(dibromomethyl)benzene (2.22 g, 5.21 mmol) in 15 mL DMSO over 30 min. Stirring at 80°C was continued for 24 hr, the mixture was poured into 200 mL of H_2O , neutralized with HCl solution, and the precipitate was filtered and dried. Yield was 1.49 g (60%), colorless crystals, m.p. $224.5\text{--}226^\circ\text{C}$ (benzene). IR bands, cm^{-1} : 1540, 1500, 1330, 1010 (Pz). NMR ^1H (DMSO- d_6), δ , ppm.: 2.09 (12H, 3- CH_3 -Pz), 2.13 (12H, 5- CH_3 -Pz), 5.90 (4H, H^d -Pz), 6.99 (4H, Ar), 7.70 (2H, Pz_2CH). Elemental analysis calculated for $C_{28}H_{34}N_8$, %: C 69.68; H 7.10; N 23.22. Found, %: C 70.01; H 7.10; N 22.90.

1,3-Bis(pyrazol-1-yl)propanedinitratocopper—[Cu(L1)(O_2NO)₂] (**1**) was prepared by adding a solution of 1,3-bis(pyrazol-1-yl)propane (0.164 g, 0.93 mmol) in 1 mL of acetone to a solution of $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (0.225 g, 0.93 mmol) in 2 mL of the same solvent. Green crystals formed upon standing for 24 hr at room temperature were filtered and dried *in vacuo*. Yield was 0.291 g (83%), m.p. 226°C (decomposed); λ , $\Omega^{-1} \cdot \text{cm}^2 \cdot \text{mol}^{-1}$: 52. Elemental analysis calculated for $C_9H_{12}CuN_6O_6$, %: C 29.72; H 3.32; N 23.10. Found, %: C 30.01; H 3.20; N 22.84. IR bands, ν cm^{-1} : 1500, 1285 (ν_3), 1384 ($\beta_{\text{C-H}}$), 1075 (Pz), 1015 (ν_1), 810 (ν_2), 749, 710 (ν_4). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L} \cdot \text{cm}^{-1} \cdot \text{mol}^{-1}$): 770 (42), 207 (28330). $E_{1/2} = -150$ mV, $\Delta E = 71$ mV.

Complexes **2–6** were prepared similarly from equimolar amounts of the corresponding ligands and Cu(II) nitrate or chloride.

1,3-Bis(3,5-dimethylpyrazol-1-yl)propanedinitratocopper—[Cu(L2)(O₂NO)₂] (2). Yield was 94%, light-green crystals, m.p. 210–211°C; λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 22. Elemental analysis calculated for C₁₃H₂₀CuN₆O₆, %: C 37.19; H 4.80; N 20.02. Found, %: C 37.25; H 4.78; N 20.10. IR bands, ν , cm^{-1} : 1555 (Pz), 1494, 1270 (ν_3), 1385 ($\beta_{\text{C-H}}$), 1050 (Pz), 1001 (ν_1), 806 (ν_2), 757, 705 (ν_4). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 800 (39), 211 (12730). $E_{1/2}$ = −139 mV, ΔE = 90 mV.

Bis[2-(pyrazol-1-yl)ethyl]etherdinitratocopper—[Cu(L3)(O₂NO)₂] (3). Yield was 88%, blue crystals, m.p. 216–217°C; λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 19. Elemental analysis calculated for C₁₀H₁₄CuN₆O₇, %: C 30.50; H 3.58; N 21.34. Found, %: C 30.50; H 3.44; N 21.15. IR bands, ν , cm^{-1} : 1521 (Pz), 1476, 1306 (ν_3), 1292 ($\beta_{\text{C-H}}$), 1095 (C–O), 999 (Pz), 813 (ν_2), 1026 (ν_1). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 750 (23), 212 (13080). $E_{1/2}$ = −149 mV, ΔE = 43 mV.

Bis[2-(3,5-dimethylpyrazol-1-yl)ethyl]etherdinitratocopper—[Cu(L4)(O₂NO)₂] (4). Yield 90%, blue crystals, m.p. 218–219°C; λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 35. Elemental analysis calculated for C₁₄H₂₂CuN₆O₇, %: C 37.37; H 4.93; N 18.68. Found, %: C 37.26; H 4.88; N 18.69. IR bands, ν , cm^{-1} : 1555 (Pz), 1482, 1296 (ν_3), 1302 (β_{CH}), 1102 (C–O), 1018 (Pz), 999 (ν_1), 826 (ν_2), 739, 710 (ν_4). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 700 (51), 217 (22520). $E_{1/2}$ = −196 mV, ΔE = 125 mV.

Bis[2-(pyrazol-1-yl)ethyl]etherdichlorocopper dihydrate—[Cu(L3)Cl₂] $\cdot$ 2H₂O (5). Yield was 74%, green crystals, m.p. 240–242°C (decomposed); λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 15. Elemental analysis calculated for C₁₀H₁₈CuCl₂N₄O₃, %: C 31.88; H 4.82; N 14.87. Found, %: C 31.57; H 4.93; N 15.02. IR bands, ν , cm^{-1} : 1518, 1420 (Pz), 1282 ($\beta_{\text{C-H}}$), 1101 (C–O), 995 (Pz). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 855 (118), 217 (5850). $E_{1/2}$ = −142 mV, ΔE = 84 mV.

Bis[2-(3,5-dimethylpyrazol-1-yl)ethyl]etherdichlorocopper—[Cu(L4)Cl₂] (6). Yield was 85%, green crystals, m.p. 203–204°C; λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 14. Elemental analysis calculated for C₁₄H₂₂Cl₂CuN₄O, %: C 42.38; H 5.59; N 14.12. Found, %: C 42.52; H 5.58; N 14.43. IR bands, ν , cm^{-1} : 1555, 1495 (Pz), 1317 ($\beta_{\text{C-H}}$), 1100 (C–O), 999 (Pz). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 720 (sh.), 375 (105), 263 (2130), 233 (12890). $E_{1/2}$ = −120 mV, ΔE = 110 mV.

μ -{1,4-Bis[bis(3,5-dimethylpyrazol-1-yl)methyl]benzene}nitratodicopper(II) nitrate—[Cu₂(μ -L5)(ONO₂)₂](NO₃)₂ (7). Yield was 88%, light-blue crystals, m.p. 225°C (decomposed). Elemental analysis calculated for C₂₈H₃₄Cu₂N₁₂O₁₂, %: C 39.21; H 4.00; N 19.60. Found, %: C 38.95; H 4.20; N 19.32. IR bands, ν , cm^{-1} : 1561, 1467 (Pz), 1385, 1321 (ν_3), 993 (Pz), 1045 (ν_1), 822 (ν_2), 709, 703 (ν_4). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 720 (78), 214 (57250), 209 (59160). $E_{1/2}$ = −267 mV, ΔE = 242 mV.

μ -1,4-Bis[bis(3,5-dimethylpyrazol-1-yl)methyl]benzenedichlorodicopper—[Cu₂(μ -L5)Cl₄] (8). Yield was 85%, yellow crystals, m.p. 222°C (decomposed); λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 27. Elemental analysis calculated for C₂₈H₃₄Cl₄Cu₂N₈, %: C 44.75; H 4.56; N 14.91. Found, %: C 44.98; H 4.80; N 14.53. IR bands, ν , cm^{-1} : 1540 (Pz), 1290 ($\beta_{\text{C-H}}$), 1020 (Pz). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 770 (131). $E_{1/2}$ = −237 mV, ΔE = 218 mV.

1,3-Bis(3,5-dimethylpyrazol-1-yl)propane-2,2'-bipyridylnitratocopper(II) nitrate—[Cu(L2)(bipy)(O₂NO)]NO₃ (9) was prepared by addition of a solution of 2,2'-bipyridyl (0.078 g, 0.5 mmol) in 0.5 mL of acetone to a solution of complex **1** (0.21 g, 0.5 mmol) in 2 mL of acetone. Crystals precipitated immediately and were filtered, washed with acetone, and dried *in vacuo*. Yield was 0.183 g (63%), light-blue crystals, m.p. 233°C (decomposed); λ , $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$: 55. Elemental analysis calculated for C₂₃H₂₈CuN₈O₈, %: C 47.95; H 4.90; N

19.45. Found, %: C 47.71; H 4.55; N 19.18. IR bands, ν , cm^{-1} : 1602 (bipy), 1566 (Pz), 1487, 1275 (ν_3), 1384 ($\beta_{\text{C-H}}$), 1023 (Pz), 1003 (ν_1), 778 (ν_2), 749, 710 (ν_4). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 720 (sh.), 312 (21210), 301 (22400), 245 (sh.), 209 (42900). $E_{1/2} = -135$ mV, $\Delta E = 101$ mV. Mixed-ligand complexes **10** and **11** were prepared similarly.

Bis[2-(pyrazol-1-yl)ethyl]ether-2,2'-bipyridylnitratocopper(II) nitrate—[Cu(L3)(bipy)(O₂NO)]NO₃ (**10**). Yield was 67%, dark-blue crystals, m.p. 198–199°C; λ , nm : 312 (21210), 301 (22400), 245 (sh.), 209 (42900). $E_{1/2} = -135$ mV, $\Delta E = 101$ mV. Found, %: C 43.38; H 3.98; N 20.33. IR bands, ν , cm^{-1} : 1609, 1600 (bipy), 1521 (Pz), 1466, 1307 (ν_3), 1363 (ν_3 of a free NO₃[−]), 1292 ($\beta_{\text{C-H}}$), 1095 (C–O), 1026 (ν_1), 999 (Pz), 813 (ν_2). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 695 (68), 311 (14660), 300 (15360), 213 (29150). $E_{1/2} = -189$ mV, $\Delta E = 121$ mV.

Bis[2-(3,5-dimethylpyrazol-1-yl)ethyl]ether-2,2'-bipyridylnitratocopper(II) nitrate—[Cu(L4)(bipy)(O₂NO)]NO₃ (**11**). Yield was 67%, blue crystals, m.p. 198–199°C; λ , nm : 313 (18720), 303 (18720), 247 (sh.), 210 (45610). $E_{1/2} = -195$ mV, $\Delta E = 120$ mV. Found, %: C 47.22; H 4.84; N 18.30. IR bands, ν , cm^{-1} : 1609, 1602 (bipy), 1555 (Pz), 1474, 1289 (ν_3), 1102 (C–O), 1017 (ν_1), 999 (Pz), 812 (ν_2), 732, 710 (ν_4). UV-Vis bands, λ_{max} , nm (ϵ , $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$): 700 (72), 313 (18720), 303 (18720), 247 (sh.), 210 (45610). $E_{1/2} = -195$ mV, $\Delta E = 120$ mV.

Computational chemistry

Starting structures of Cu(II) complexes for DFT geometry optimizations were generated using HyperChem 8 model builder, and preliminary optimization was performed using the PM3 method.²² Doublet-state gas phase geometry optimizations of the resulting structures were carried out at the DFT level of theory employing the three-parameter hybrid B3LYP functional^{23–25} and 6-31G(d) basis set²⁶ in the Gaussian 03W package.²⁷ Frequency calculations were performed for all molecules in order to establish the nature of the stationary points. Lack of imaginary vibration modes for all of the optimized structures indicate that the stationary points found corresponded to minima on the potential energy surface. The atomic coordinates for all of the optimized structures are provided in Supplementary Table S1.

In order to obtain more accurate electronic structure information, single-point DFT calculations for optimized structures of Cu(II) complexes were performed using the ORCA 2.6.35 package developed by F. Neese.²⁸ The calculations utilized BP86 functional²⁹ and Ahlrichs' valence triple-dzeta basis set³⁰ with two sets of polarization functions (TZVPP) on all atoms [The Ahlrichs (2df,2pd) polarization functions were obtained from the TurboMole basis set library under ftp.chemie.uni-karlsruhe.de/pub/basen]. The Coulomb's integrals were evaluated employing the resolution of identity approximation. The TZV/J basis set^{31,32} was used as an auxiliary set.

Evaluation of SOD-like activity

O₂^{•−} was generated using a nonenzymatic system in the presence or absence of test compounds, and O₂^{•−} was detected by monitoring reduction of nitroblue tetrazolium (NBT) to monoformazan dye at 560 nm. The system contained 3 μM phenazine methosulfate (PMS), 200 μM NADH, and 50 μM NBT in 0.05 M phosphate buffer (pH 7.5).³³ The reactions were monitored at 560 nm with a SpectraMax Plus microplate spectrophotometer at 25°C, and the rate of absorption change was determined. The concentration required to produce 50% inhibition (IC₅₀) was obtained by graphing the rate of NBT reduction versus the logarithm of the concentration of the copper compound. The kinetics constant k_{cat} was calculated using the equation $k_{\text{cat}} = k_{\text{NBT}}[\text{NBT}]/\text{IC}_{50}$, where $k_{\text{NBT}} = 5.94 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ is the second-order rate constant for NBT.³⁴

Phagocyte isolation

Human blood was collected from healthy volunteers into heparin-containing Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of red blood cells, as described previously.³⁵ Isolated neutrophils were washed twice and resuspended in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} . Neutrophil preparations were routinely >95% pure, as determined by light microscopy, and >98% viable, as determined by trypan blue exclusion.

Murine bone marrow cells were flushed from tibias and femurs of BALB/c mice with HBSS using a syringe with 27-gauge needle. The cells were resuspended by gentle pipetting and filtered through 70 μm nylon cell strainers (Becton Dickinson). This procedure minimizes any phagocyte activation during isolation and, thus, their function more accurately reflects the physical condition of these cells *in vivo*. ROS-generation in bone marrow phagocyte preparations is primarily due to neutrophils and macrophages.³⁶

Phagocyte ROS production

ROS production in cell suspensions of murine bone marrow phagocytes or human neutrophils was determined by monitoring L-012-enhanced luminescence, which represents a sensitive and reliable method for detecting $\text{O}_2^{\bullet-}$ production in *in vitro* and *ex vivo* systems.³⁷ Cell suspensions (10^5 cells/mL) were pretreated with Cu(II) complexes or ethanol vehicle for 5 min, and luminescence was monitored after treatment of the cells with (stimulated ROS production) or without (spontaneous ROS production) 100 nM phorbol myristate acetate (PMA) in the presence of 50 μM L-012. Intercellular ROS production was also monitored after treatment of the neutrophils with serum-opsonized zymosan particles (100 $\mu\text{g/mL}$) for 15 min. In this case L-012-dependent luminescence was measured in the presence of 50 μM L-012 and bovine erythrocyte SOD (5 U/mL).¹³ Luminescence was monitored for 60 min (2 min intervals) at 37°C using a Fluroscan Ascent FL microtiter plate reader (Thermo Electron, Waltham, MA). The curve of light intensity (in relative luminescent units) was plotted against time, and the area under the curve was calculated as total luminescence. The % inhibition of luminescence = (control-sample)/control $\times$ 100. IC_{50} values were obtained by graphing the % inhibition of luminescence versus the logarithm of concentration of tested compound. Each line was determined using 5–7 tested concentrations.

Cell proliferation and cytotoxicity assay

Mouse macrophage J774.A1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) bovine serum, 100 $\mu\text{g/mL}$ streptomycin, and 100 U/mL penicillin, and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 . The cells were grown to confluence and gently detached by scraping.

Cell proliferation and cytotoxicity were analyzed by measuring ATP with the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. J774.A1 cells (2.5×10^4 cells per well) were suspended in phenol red-free DMEM supplemented with 3% (v/v) bovine serum, 100 $\mu\text{g/mL}$ streptomycin, and 100 U/mL penicillin, and seeded in 96-well microtiter plates (Costar, Milpitas, CA, USA). Plated cells were treated with test compounds for 18 hr. At 30 min before the end of the treatment, the cells were allowed to equilibrate to room temperature, substrate was added, and the samples were analyzed with a Fluroscan Ascent FL microplate reader.

Results and discussion

Synthesis of complexes

To study the influence of Cu(II) coordination sphere structure on $O_2^{\bullet-}$ scavenging activity of copper complexes, we prepared compounds with different Cu(II) environments. Complexes **1** and **2** with $\{CuN_2O_4\}$ cores were obtained by the reaction of one equivalent of bidentate 1,3-bis(pyrazol-1-yl)propane ligands (L1, L2, Scheme 1) with Cu(II) nitrate in acetone solution. In addition, we obtained Cu(II) nitrate and chloride complexes with potentially tridentate bis[2-(pyrazol-1-yl)ethyl]ether ligands (L3, L4, Scheme 1) containing additional donor oxygen atoms in the spacer between pyrazole rings. As demonstrated by spectroscopic investigation and X-ray diffraction analysis, these complexes have $\{CuN_2O_5\}$ (**3**, **4**) and $\{CuN_2OCl_2\}$ (**5**, **6**) cores.

Binuclear Cu(II) $\{CuN_2O_4\}_2$ and $\{CuN_2Cl_2\}_2$ complexes **7** and **8** were prepared by the reaction of two equivalents of Cu(II) salts with the bitopic 1,4-bis[bis(3,5-dimethylpyrazol-1-yl)methyl]benzene ligand (L5, Scheme 1) containing two bis(pyrazol-1-yl)methane units in the same molecule.

In order to modify the structure of the coordination center even further, we prepared mixed-ligand complexes containing both bis(pyrazole) and 2,2'-bipyridyl ligands in the coordination sphere. Mixed-ligand complexes **9**, **10**, and **11** were obtained by reacting bis(pyrazole)-Cu(II) nitrate complexes **2**, **3**, and **4** with one equivalent of 2,2'-bipyridyl in acetone solution (Scheme 2). When Cu(II) chloride complexes were introduced into the reaction with 2,2'-bipyridyl, bis(pyrazole) ligands were displaced from the coordination sphere, and only homoligand $[Cu(bipy)Cl_2]$ species could be isolated. Mixed-ligand complexes have $\{CuN_4O_2\}$ (**9**) or $\{CuN_4O_3\}$ (**10**, **11**) cores depending on bis(pyrazole) ligand used.

All of the coordination compounds obtained gave satisfactory elemental analysis and were characterized by molar conductivity, IR and electronic spectroscopy, and cyclic voltammetry. Additionally, X-ray structure analysis was performed for complex **4**.

Cu(II) chloride complexes **5** and **6** are non-electrolytes in acetone solution, indicating that both chloride anions are bound to the coordination center. Nitrate complexes **1-4** are also weak electrolytes, suggesting that both nitrate ions are included in the coordination sphere. Although molar conductivity for complex **1** ($52 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$) was somewhat higher than one could expect for a neutral complex, it is still lower than the average value of $120 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ for 1:1 electrolytes in acetone.³⁸ Mixed-ligand complexes **9-11** with 2,2'-bipyridyl demonstrate molar conductivity values close to those reported for 1:1 electrolytes,³⁸ indicating that one of nitrate ions is displaced from the coordination sphere by the 2,2'-bipyridyl ligand, while the other remains coordinated (Scheme 2).

X-ray crystal structure of complex **4**

Bis[2-(pyrazol-1-yl)ethyl]ether ligands are known to be coordinated in either a bidentate (via two pyrazole nitrogen atoms) or tridentate fashion (with additional coordination through ether oxygen atom).³⁹ In order to determine the coordination mode of L3 and L4 ligands in the complexes reported here, we have investigated the crystal structure of one complex, namely $[Cu(L4)(NO_3)_2]$ (**4**).

Complex **4** crystallizes in a monoclinic crystal system, and each unit cell contains four formula units of the complex. The structure of the complex is shown in Figure 1, and selected bond distances and angles are given in Table 1. The complex consists of discrete neutral $[Cu(L4)(O_2NO)_2]$ molecules. The ligand L4 is coordinated in a tridentate T-shaped fashion via two nitrogen atoms of pyrazole rings and an ether oxygen atom. A similar coordination mode of

this ligand has been reported previously for Cu(I), Zn(II), and Co(II) complexes.⁴⁰⁻⁴¹ Overall, the Cu(II) ion in complex **4** is hepta-coordinated, with the four remaining coordination sites being occupied by oxygen atoms of the two bidentate nitrate ions. Two pyrazole nitrogen atoms, two nitrate oxygen atoms, and the copper atom are nearly planar, with maximum deviation of atom positions from the least squares plane being only 0.104 Å. Above that plane lies a coordinated ether oxygen atom, with the Cu–O bond length being equal to 2.306(1) Å. Below the plane, two other nitrate oxygen atoms are located at a distance of 2.68–2.69 Å from the copper center. The whole coordination environment of Cu(II) ion thus forms an augmented triangular prism.

It should be noted that the Cu–O bonding distances for bidentate nitrate ions significantly differ from each other, with one of the bond lengths being equal to 1.99–2.03 Å, while the other is 2.68–2.69 Å. Kleywegt et al.⁴² suggested the use of two criteria for assigning nitrate coordination modes: difference between Cu–O distances (d_2-d_1) and CuON angles ($\theta_1-\theta_2$). Using these criteria, a coordination mode intermediate between unidentate and anisobidentate can be assigned for both nitrate ions. One is coordinated in a fashion more closely resembling anisobidentate ($d_2-d_1 = 0.662$ Å, $\theta_1-\theta_2 = 27.56$ deg.; the literature criteria for anisobidentate coordination is 0.3–0.6 Å and 14–28 deg.⁴²). The other unidentate coordination mode is more typical ($d_2-d_1 = 0.691$ Å, $\theta_1-\theta_2 = 33.23$ deg., for the unidentate coordination these criteria are greater than 0.6 Å and 28 deg.⁴²). Such intermediate nitrate coordination by Cu(II) ions has been reported previously. For example, in the Cu(II) complex with bis[2-(3,5-dimethylpyrazol-1-yl)ethyl]amine,⁴³ Cu–O distances [2.102(8) and 2.540(8) Å] were found to be very close to the values reported here for the L4 ligand.

IR and UV-Vis spectroscopy

In Cu(II) nitrate complexes, NO_3^- ions may be coordinated in a mono- or bidentate fashion, and the coordination modes of nitrate ions can be established by means of IR-spectroscopy.⁴⁴⁻⁴⁷ In the IR spectra of Cu(II) nitrate complexes, two intense bands in the 1470–1500 and 1270–1300 cm^{-1} regions (Table 2) are assigned to N–O stretching vibrations (ν_3) in coordinated NO_3^- ion having C_{2v} symmetry.⁴⁵ The bands of in-plane bending vibrations (ν_4) appear at 705–710 and 730–760 cm^{-1} . Large splitting of these bands ($\Delta(\nu_3) > 180$ and $\Delta(\nu_4) > 22$ cm^{-1}) confirms the presence of bidentate nitrate ions in all four complexes. For monodentate coordination of NO_3^- , splitting of the ν_3 band is not higher than 120 cm^{-1} , and that of ν_4 is less than 20 cm^{-1} .⁴⁵ Complex **7** demonstrated much smaller values of $\Delta(\nu_3)$ and $\Delta(\nu_4)$ splitting (64 and 6 cm^{-1} , respectively), which is characteristic of monodentate nitrate ions.

Single bands around 820 cm^{-1} (out-of-plane bending vibrations ν_2),⁴⁵ as well as bands of medium intensity at about 1000 cm^{-1} (symmetric stretching vibrations ν_1),⁴⁵ were observed in IR-spectra of the nitrate complexes (Table 2). The occurrence of the latter band gives further evidence for nitrate ion coordination, since the vibrational transition ν_1 in free NO_3^- is symmetry-forbidden, and this band does not appear in the IR spectra or has negligible intensity.⁴⁴

Bands due to pyrazole ring vibrations in the IR spectra of the complexes are shifted relative to the corresponding bands in the spectra of free ligands. In the spectra of the mixed-ligand complexes **9–11**, additional bands due to 2,2'-bipyridyl ligand vibrations were detected.

Bis[2-(pyrazol-1-yl)ethyl]ether ligands are able to adopt two coordination modes, i.e. $\kappa^2\text{-N,N}$ -bidentate or $\kappa^3\text{-N,N,O}$ -tridentate. As shown by X-ray analysis for compound **4**, the ligand in this complex exhibits a $\kappa^3\text{-N,N,O}$ -tridentate coordination to the Cu(II) ion. In the IR spectrum of the free ligand, the C–O stretching vibration band is located at 1113 cm^{-1} , whereas in the spectrum of complex **4**, this band is shifted towards lower frequencies by 11 cm^{-1} (Table 2). Similarly, in the IR spectra of other bis(pyrazol-1-yl)ether complexes, the C–O stretching bands

undergo a coordination-induced lower-frequency shift of about 10 cm^{-1} . In case of $\kappa^2\text{-N,N}$ -bidentate coordination of the ligand, less significant C-O band shifts should be expected since the oxygen atom is not bound to copper and its vibrational motion should be less affected by a ligand coordination. Therefore, based on X-ray analysis and IR spectroscopy, we may assume a κ^3 -tridentate coordination of bis[2-(pyrazol-1-yl)ethyl]ether ligands with two pyrazole nitrogen atoms and an ether oxygen bound to the Cu(II) center in all of the prepared complexes (**3-6** and **10-11**).

The Cu(II) complexes studied here exhibit d-d* absorption bands in diverse regions in accordance with their different coordination cores. Thus, hexacoordinated complexes (**1**, **2**, and **9**) show single absorption bands in the 720-800 nm region, which is characteristic of a distorted octahedral structure.⁴⁸ Compounds with CuN_2Cl_2 coordination centers (**8** and **12**) demonstrate bands at 860 and 770 nm, which are usually found in the spectra of tetrahedral Cu(II) complexes.⁴⁸

Penta-coordinated complexes **5** and **6** of bis(pyrazole)ether ligands have single absorption bands at 855 and 720 nm, which is in good agreement with literature values for a square pyramidal Cu(II) arrangement.⁴⁸ In the spectra of hepta-coordinated Cu(II) nitrate complexes with bis(pyrazolyl)ether ligands **3** and **4**, as well as mixed ligand complexes **10** and **11**, d-d* absorptions undergo a shift towards shorter wavelengths compared to hexacoordinated octahedral species.

In the UV region, bands at 210-220 nm were assigned to bis(pyrazole) intraligand excitations. In the spectra of mixed ligand complexes, two additional bands were detected near 300 and 310 nm, which are due to bipy intraligand transitions. Intraligand excitation bands undergo a red shift relative to bands in the spectra of free ligands. In addition, shoulder bands at 245 nm detected in the spectra of mixed ligand complexes can be assigned to metal-to-ligand charge transfer excitations.

It should be noted that positions of the absorption bands and, more importantly, values of extinction coefficients are different for mixed-ligand and homoligand complexes. Thus, it is evident that mixed-ligand complexes are indeed individual compounds and not mixtures of homoligand Cu(II)-bis(pyrazolyl) and Cu(II)-bipyridyl complexes.

SOD-like activity of the complexes

The $\text{O}_2^{\bullet-}$ scavenging activities (IC_{50} and k_{cat}) of the Cu(II) complexes determined in a nonenzymatic (PMS/NADH) $\text{O}_2^{\bullet-}$ -generating system are shown in Table 3. In order to expand the range of complexes studied and explore the influence of ligand on SOD activity, we also included some previously reported bis(pyrazol-1-yl)methane-Cu(II) complexes²⁰ (designated as **12-18**) for comparison in our current study (Table 3). We found that all five bipy-Cu(II) complexes demonstrated high SOD-like activity, with IC_{50} values in the low micromolar range (0.8-1.7 μM). Although the bipy complexes were not as effective as horseradish SOD (Table 3) or bovine erythrocyte SOD ($\text{IC}_{50}=4\times 10^{-8}$)⁴⁹, their activities were on the same order of magnitude as the best SOD analogs described in the literature.⁵⁰⁻⁵² The lower k_{cat} of Cu(NO_3)₂ compared with that of the bipy complexes suggests that the activity of the complexes was not due to their dissociation in solution. It should be noted that ligands (L1-L7) did not exhibit any $\text{O}_2^{\bullet-}$ scavenging effects over the same concentration range (0.1–100 μM) in the nonenzymatic $\text{O}_2^{\bullet-}$ -generating system (data not shown).

Electrochemistry

In order to gain some insight into the possible reasons for increased SOD-like activity of bipyridyl mixed-ligand complexes, their redox behavior was studied by cyclic voltammetry.

Redox properties of Cu(II) complexes are known to correlate with their $O_2^{\bullet-}$ scavenging activity.⁵³⁻⁵⁴ All of the investigated homoligand complexes demonstrated quasi-reversible one-electron reduction waves in the potential range of -111 to -214 mV vs. Ag/AgCl electrode, which can be assigned to the Cu(II)/Cu(I) pair (Table 4). Separations of cathodic and anodic peaks $\Delta E = E_{p.a.} - E_{p.c.}$ are in the range reported for other SOD mimetics¹², thus indicating that a catalytic $O_2^{\bullet-}$ dismutation cycle is possible for these complexes. It is interesting to note that mixed-ligand bipy complexes exhibited $E_{1/2}$ values at the negative end of the potential range found for all the complexes investigated. Nevertheless, these compounds exhibited the highest SOD activity among the complexes. For three of the mixed-ligand complexes (**9-11**), reduction potential values were very close to those of their non-bipy precursors (**2-4**), although the bipy complexes were significantly more active $O_2^{\bullet-}$ scavengers.

Although all of the complexes met the electrochemical criterion for SOD mimetics,¹² some demonstrated no SOD-like activity. Thus, the antiradical properties of the investigated complexes might be impacted by factors other than redox properties (e.g., electronic structure).

DFT study of the electronic structure of Cu(II) complexes

We investigated the electronic structure of mixed-ligand complexes together with their homoligand precursors by means of DFT calculations. Since the conversion of $O_2^{\bullet-}$ to O_2 involves $O_2^{\bullet-}$ -Cu(II) electron transfer, SOD-like activity of Cu(II) complexes is influenced by their lowest unoccupied molecular orbital (LUMO) energy.⁵⁵ Boundary orbital energies of the complexes were obtained by single-point DFT calculations. Geometries for single-point calculations were obtained by gas-phase geometry optimizations using the 6-31G(d) basis set and B3LYP functional. Coordination bond lengths and angles obtained by DFT geometry optimization of complex **4** are fairly close to those determined by X-ray crystallography (Table 1), one exception being the Cu-O(1) bond between the bis(pyrazole) ligand oxygen atom and copper center, which is predicted to be too long. It should be noted that essentially the same geometrical parameters were obtained when the optimization was started from the X-ray structure of complex **4** instead of PM3 geometry. Therefore, the differences in the calculated gas-phase and X-ray crystal structures are likely due to packing effects. A good qualitative agreement between calculated and experimental nitrate ion coordination geometries in complex **4** should be noted. One of the bonds in both of the nitrate ions is predicted to be about 0.63 Å longer than the other, which is in good agreement with the X-ray structure of complex **4** (0.68-0.69 Å). Therefore, the chosen B3LYP functional and 6-31G(d) basis set can be considered suitable for geometry optimizations. In order to obtain more accurate information of electronic density distribution in the complexes, a larger triple-dzeta TZVPP basis set was chosen for single-point calculations.

The values of LUMO energies obtained are shown in Table 4. These orbitals are localized on the copper atom to a significant extent, both for mixed-ligand and their homoligand counterparts (Figure 2), which should assist in $O_2^{\bullet-}$ -Cu(II) electron transfer. This transfer involves the relocation of an electron from the highest occupied orbital of $O_2^{\bullet-}$ to the lowest occupied orbital (LUMO) of the Cu(II) complex. The larger the gap between these orbitals, the more favorable is the electron transfer. Introduction of a bipyridyl ligand into the complexes dramatically lowers the LUMO energy level (by about 3 eV), which explains the increased SOD-like activity of these complexes compared to non-bipy species. The effect of the bipyridyl co-ligand on the electronic structure of bis(pyrazolyl)-Cu(II) complexes is probably explained by its known electron-withdrawing properties, leading to $d\pi-p\pi$ metal-to-ligand back-donation.⁵⁶ It should be noted that a similar effect of the bipyridyl ligand on the catalase-like activity of Cu(II) complexes was reported by Sigel et al.⁵⁷ and was explained by the increased stability of the intermediate Cu(II)-peroxo complexes and enhanced rate of an electron transfer.

Deverux with co-authors⁵⁸ reported the SOD-like activity of aliphatic Cu(II) carboxylates and their mixed-ligand bipy complexes. The introduction of a bipy ligand into the complexes had no significant influence on SOD-like activity of the carboxylate complexes,⁵⁸ which is in contrast to our current study. The differing effects of bipy ligand on SOD-like activity of carboxylate and bis(pyrazole) Cu(II) complexes is probably explained by their different coordination environment.

Antiradical activity of the bipy complexes in a cellular system

Five bipy-Cu(II) complexes and Cu(NO₃)₂ were tested for antiradical activity in a cellular system, as these Cu(II) complexes demonstrated the highest SOD-like activity in the nonenzymatic PMS/NADH system. We evaluated the ability of the complexes to scavenge ROS generated by human neutrophils, which generate significant levels of ROS through NADPH oxidase activation.⁵⁹ In addition, we also evaluated scavenging of ROS generated by murine bone marrow phagocytes, in which ROS are primarily generated by neutrophils and macrophages.⁶⁰ Most comparative studies on phagocyte isolation techniques have shown either activation or functional impairment of the cells is due to different separation processes.⁶¹ Thus, murine whole bone marrow (BM) cells represent an *ex vivo* model of cellular ROS production where cell separation was minimal.

Addition of the Cu(II) compounds resulted in a concentration-dependent inhibition of spontaneous (non-stimulated cells) and PMA-induced ROS production in both cell systems, indicating these complexes were effective scavengers of ROS generated by both murine and human phagocytes. IC₅₀ values for all bipy-Cu(II) complexes under investigation and Cu(NO₃)₂ are indicated in Table 5. To evaluate intracellular SOD-like activity, cells were incubated with opsonized zymosan for 15 min, and chemiluminescence was measured in the presence of SOD (5 U/mL) and the indicated concentrations of Cu(II) compounds. In this system, O₂^{•-} is generated in intracellular phagosomes,⁶² whereas any extracellular O₂^{•-} will be removed by SOD. Again, bipy-Cu(II) complexes were active in scavenging intracellular ROS (Table 5). Although the bipy-Cu(II) complexes scavenged ROS at nanomolar concentrations, complex **9** was most active compound in these cellular systems.

Bovine serum albumin (BSA) is one of strongest biological chelators of cupric ions and can inhibit their SOD-like activity.¹² Thus, SOD-like activity of our Cu(II) complexes was also evaluated when increasing concentrations of BSA were added to the system containing PMA-stimulated human neutrophils. We found that increasing concentrations of BSA significantly inhibited the SOD-like activity of Cu(NO₃)₂ but had a much lower effect on the bipy-Cu(II) complexes tested (Figure 3). These data indicate that bipy-co-ligands and L2-L4, L6 and L7 ligands are stronger chelators of Cu(II) ions than BSA and that the bipy-Cu(II) complexes are highly stable.

Qualification of an SOD mimic

To act as a qualified SOD mimic (*in vitro*), a compound needs to have the following properties⁸: (1) the compound should have a relatively long metabolic half-life for it to carry out its SOD-like activity, (2) it should be able to penetrate into the cells to reach the target region, and (3) it should not be toxic at the concentrations needed for SOD-like activity. The data presented here demonstrate that the bipy Cu(II) complexes meet qualifications (1) and (2). Indeed, significant differences between the UV-Vis spectra of the ligands and Cu(NO₃)₂ versus the Cu(II) complexes suggest stability of the complexes under experimental conditions is rather high. In addition, stability of the complexes was also confirmed by recording their UV spectra at given intervals of time over several hours and under conditions close to those used for evaluating SOD-like activity: 10 μM concentration in 0.05 M phosphate buffer (pH 7.5). No significant change in absorbance values or band shapes were observed in the spectra

of all five mixed-ligand complexes for at least 4 hr (data not shown), indicating that they are quite stable under the experimental conditions. In addition, positions of the absorbance bands (two bands near 300 and 310 nm, shoulder at 245 nm) as well as extinction coefficients (1.4×10^4 – 1.5×10^4 L·cm⁻¹·mol⁻¹) in phosphate buffer were very close to those obtained in ethanol solutions of the mixed-ligand complexes, thus indicating that the structure of their coordination sphere remains intact when changing from ethanol to aqueous buffer. In cell-based assay systems, the bipy complexes exhibited much higher SOD-like activity as compared to Cu(NO₃)₂, again indicating the complexes retain their structural integrity in a biologically-relevant environment. We also demonstrated that the mixed complexes (**9–13**) can penetrate human neutrophils and scavenge intracellular ROS in zymosan-stimulated neutrophils in the presence of external SOD. To address requirement (3), we performed a cell viability/proliferation test on J774.A1 macrophages treated with selected bipy-complexes. Proliferation and viability of J774.A1 cells were not affected by treatment of the cells with complexes **10–13** over a wide concentration range (3–25 μM), as no significant differences from the control were observed ($P > 0.05$) (Figure 4).

ROS play an important role in the pathogenesis of a number of diseases, and recent advances in the development of SOD mimetics suggest that such compounds could be used as therapeutic agents in these diseases.⁶³ Indeed, several Cu(II) complexes with SOD-like activity have been shown to improve disease status in animal models of free-radical tissue damage, such as radiation-induced skin damage, alloxan-induced diabetes, and aging-associated cardiovascular dysfunction.^{64–66} Since our results indicate that bipy mixed-ligand complexes are stable and have good qualities as SOD mimetics in enzymatic and cell-based systems, these compounds represent ideal leads for further development. We anticipate that further chemical modification of these lead SOD-mimetics could result in the development of compounds that are suitable for testing in animal models of ROS damage.

Conclusion

In the present report, we describe synthesis and characterization of eleven novel bis(pyrazole) Cu(II) complexes, including three mixed ligand 2,2'-bipyridyl complexes. Based on X-ray analysis and IR spectroscopy, a κ³-tridentate coordination of bis[2-(pyrazol-1-yl)ethyl]ether ligands with two pyrazole nitrogen atoms and ether oxygen bound to Cu(II) center was established in these complexes. All of the bipy-Cu(II) complexes demonstrated high SOD-like activity toward ROS generated chemically or by activated phagocytes. Interestingly, DFT calculations showed that introduction of a bipyridyl ligand into the complexes dramatically lowered the LUMO energy level, which explains the increased SOD-like activity of these complexes compared to non-bipy species. This work demonstrates that bipy mixed-ligand complexes may be a promising class of SOD mimetics with therapeutic potential and supports the further evaluation of these compounds in animal experimental protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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L1 (R = H); L2 (R = CH₃) L3 (R = H); L4 (R = CH₃)

L5

L6 (R = H); L7 (R = CH₃)

Scheme 1.

Scheme 2.

Figure 1. Representation of the molecular structure of complex **4** determined by X-ray crystallography. Thermal ellipsoids are drawn at 50% probability level, hydrogen atoms are omitted for clarity. Labels for symmetry equivalent atoms are not shown.

Figure 2.

Lowest unoccupied molecular orbital (LUMO) isosurfaces for Cu(II) complexes with (right column) and without (left column) additional 2,2'-bipyridyl ligands studied by density functional theory (DFT).

Figure 3. SOD-like activity of bipy-complexes and Cu(NO₃)₂ on ROS produced by activated human neutrophils in the presence of bovine serum albumin (BSA) as a copper ion scavenger. The data are expressed as means ±SD of triplicate samples from three experiments.

Figure 4. Effect of bipy-complexes **9-11**, **17**, and **18** on cell viability. The data are expressed as means $\pm$ SD of triplicate samples from three experiments.

Table 1

Selected bond distances and angles determined by X-ray crystallography and DFT geometry optimization for complex [Cu(L4)(O₂NO)₂] (**4**)

Bond	Distance, Å	Calc.	Angle	deg.	Calc.
Cu–N(1)	2.006(1)	2.052	N(1)–Cu–N(1)	174.55(5)	159.53
Cu–O(1)	2.306(1)	2.557	O(2)–Cu–N(1)	89.68(4)	90.9
Cu–O(2)	2.007(1)	1.949	O(2)–Cu–O(2)	173.87(4)	178.3
Cu–O(4)	2.681(1)	2.580	O(1)–Cu–O(2)	93.06(4)	90.9
			N(1)–Cu–O(1)	87.27(5)	79.8

Table 2
Frequencies of characteristic vibrations in the IR-spectra of the Cu(II) complexes

No.	Formula	ν_1	ν_2	ν_3	$\Delta(\nu_3)$	ν_4	$\Delta(\nu_4)$	$\nu(\text{C-O})$	$\Delta\nu(\text{C-O})^a$
1	[Cu(L1)(O ₂ NO ₂) ₂]	1015	810	1500 1285	215	749 710	39	–	–
2	[Cu(L2)(O ₂ NO ₂) ₂]	1001	–	1494 1270	224	757 705	52	–	–
3	[Cu(L3)(O ₂ NO ₂) ₂]	1026	813	1476 1306	170	–	–	1095 ^b	10
4	[Cu(L4)(O ₂ NO ₂) ₂]	999	826	1482 1296	186	739 710	29	1102 ^c	11
5	[Cu(L3)Cl ₂]	–	–	–	–	–	–	1101 ^b	11
6	[Cu(L4)Cl ₂]	–	–	–	–	–	–	1100 ^c	–
7	[Cu ₂ (μ -L5)(ONO ₂) ₂](NO ₃) ₂	1045	822	1385 1321	64	709 703	6	–	–
9	[Cu(L2)(bipy)(O ₂ NO)]NO ₃	1003	778	1487 1275	212	749 710	39	–	–
10	[Cu(L3)(bipy)(O ₂ NO)]NO ₃	1026	813	1466 1307 1363	159	–	–	1095 ^b	10
11	[Cu(L4)(bipy)(O ₂ NO)]NO ₃	1017	812	1474 1289	185	732 710	22	1102 ^c	11

(–) band was not detected.

^a $\Delta\nu(\text{C-O}) = \nu(\text{C-O})_{\text{complex}} - \nu(\text{C-O})_{\text{ligand}}$;

^b 1085 cm^{–1} in the spectra of free ligand;

^c 1113 cm^{–1} in the spectra of free ligand.

Table 3
SOD-like effect of Cu(II) compounds in the phenazine methosulfate (PMS)/NADH system and cyclic voltammetry characteristics

No.	Formula	PMS/NADH system		$E_{1/2}$ (mV)	ΔE (mV)
		IC_{50} (μM) ^a	k_{cat} ($M^{-1} s^{-1}$) ^a		
1	[Cu(L1)(O ₂ NO ₂)]	55.8±20.8	5.0×10^4	-150	71
2	[Cu(L2)(O ₂ NO ₂)]	73.2±18.1	4.0×10^4	-139	90
3	[Cu(L3)(O ₂ NO ₂)]	24.7±2.7	1.2×10^5	-149	43
4	[Cu(L4)(O ₂ NO ₂)]	N.A.	N.A.	-196	125
5	[Cu(L3)Cl ₂]	26.0±12.9	1.1×10^5	-142	84
6	[Cu(L4)Cl ₂]	N.A.	N.A.	-120	110
7	[Cu ₂ (μ -L5)(ONO ₂) ₂](NO ₃) ₂	26.1±6.2	1.1×10^5	-267	242
8	[Cu ₂ (μ -L5)Cl ₄]	13.4±5.3	2.2×10^5	-237	218
9	[Cu(L2)(bipy)(O ₂ NO)]NO ₃	1.3±0.5	2.3×10^6	-135	101
10	[Cu(L3)(bipy)(O ₂ NO)]NO ₃	0.8±0.2	3.7×10^6	-189	121
11	[Cu(L4)(bipy)(O ₂ NO)]NO ₃	0.9±0.5	3.3×10^6	-195	120
12	[Cu(L6)Cl ₂]	35.3±12.2	8.4×10^4	-169	128
13	[Cu(L6) ₂]Cl ₂	21.1±9.5	1.4×10^5	-111	45
14	[Cu(L7)Cl ₂]	52.3±12.3	5.7×10^4	-179	140
15	[Cu(L7) ₂]Cl ₂	33.3±4.7	8.9×10^4	-202	140
16	[Cu(L6)(O ₂ NO ₂)]	N.A.	N.A.	-214	182
17	[Cu(L6)(bipy)(O ₂ NO)]NO ₃	1.0±0.4	3.0×10^6	-153	121
18	[Cu(L7)(bipy)(O ₂ NO)]NO ₃	0.9±0.4	3.3×10^6	-190	157
	Cu(NO ₃) ₂	4.9±1.4	6.1×10^5		
	CuCl ₂	9.5±5.1	3.1×10^5		
	Horseradish SOD	0.07±0.01	4.2×10^8		

N.A., no activity over the concentration range tested.

^aThe data are expressed as means ±SD of triplicate samples from three experiments.

Table 4

Density functional theory (DFT) electronic structure calculations of Cu(II) complexes with and without additional 2,2'-bipyridyl ligands

Non-bipy complexes			Bipy complexes		
No.	Formula	E(LUMO) (eV)	No.	Formula	E(LUMO) (eV)
2	[Cu(L2)(O ₂ NO ₂)]	-4.402	9	[Cu(L2)(bipy)(O ₂ NO)]NO ₃	-7.138
3	[Cu(L3)(O ₂ NO ₂)]	-4.133	10	[Cu(L3)(bipy)(O ₂ NO)]NO ₃	-7.178
4	[Cu(L4)(O ₂ NO ₂)]	-3.988	11	[Cu(L4)(bipy)(O ₂ NO)]NO ₃	-7.180
16	[Cu(L6)(O ₂ NO ₂)]	-4.504	17	[Cu(L6)(bipy)(O ₂ NO)]NO ₃	-7.273

Table 5
Evaluation of antiradical activity of selected Cu(II) bipy complexes on phagocyte-generated ROS

Compound number	Formula	IC ₅₀ (nM) ^a					
		Murine bone marrow leukocytes		Human neutrophils			
		Spontaneous	PMA-stimulated	Spontaneous	PMA-stimulated	Zymosan-stimulated ^b	
9	[Cu(L2)(bipy)(O ₂ NO)]NO ₃	4.3±0.3	1.3±0.1	6.7±0.8	5.3±0.4	266±25.9	
10	[Cu(L3)(bipy)(O ₂ NO)]NO ₃	9.7±0.8	6.8±0.7	9.7±1.1	6.9±0.7	315±32.3	
11	[Cu(L4)(bipy)(O ₂ NO)]NO ₃	8.3±0.7	5.3±0.4	11.1±0.9	7.9±0.8	367±34.5	
17	[Cu(L6)(bipy)(O ₂ NO)]NO ₃	22.1±3.2	19.4±1.6	11.5±1.3	8.9±0.9	388±33.7	
18	[Cu(L7)(bipy)(O ₂ NO)]NO ₃	7.5±0.6	8.3±0.7	10.3±1.2	6.9±0.5	308±29.1	
	Cu(NO ₃) ₂	10.6±1.1	1.8±0.2	12.7±1.4	12.3±1.4	934±10.1	

^aThe data are expressed as means ±SD of triplicate samples from three experiments.

^bSOD was present to scavenge any extracellular ROS.¹³