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Superoxide Dismutase Mimetic Activity of Cu(II)-Salicylic Acid Analogs

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Abstract

The superoxide dismutase (SOD)-mimetic activities of copper complexes of a series of salicylic acid (SA) analogs were tested and compared to the activity of bovine erythrocyte SOD using ferricytochrome c reduction assay. Stability constants of copper complexes were measured potentiometrically using SCOGS2 program. In the presence of 10 *g/l* albumin, all the copper complexes lost their SOD mimetic activities. Multiple regression analysis was employed for the statistical comparisons between the SOD mimetic activity and their physicochemical properties. Correlation exists for the SOD mimetic activity and steric parameter (E_s) and/or electronic parameter ($\sum\sigma$) in xanthine/xanthine oxidase (XOD) system, demonstrating that E_s plays a key role in SOD activity whereas $\sum\sigma$ influences it to a lesser extent. The protective effect of copper complexes against membrane damage was measured by counting D-glucose released frm EGs. D-glucose and XOD were entrapped within EGs and acetaldehyde was used as a substrate for XOD. In this membrane model system using EGs, hydrophobic parameter ($\sum\pi$) is of most importance, producing parabolic equation while E_s and $\sum\sigma$ appear to play a minor role in protection against D-glucose release. In summary, to design an efficient SOD mimetic, stability, steric factor, lipophilicity and redox potential should be considered.

1. INTRODUCTION

The theory of oxygen toxicity proposed by Fridovich[1], stating that superoxide anion ($O_2\cdot^-$) is the toxic species in oxygen toxicity and SOD provides marked protection against oxygen toxicity by destroying potentially harmful $O_2\cdot^-$, has received general acceptance. Oxygen free radicals have been linked to the pathophysiology of various disease states[2] and clinical applications of SOD for these diseases have been reported.[3,4]

Several authors have observed possible use of SOD as an antiinflammatory drug.[5,6] It may find applications in radioprotrection,[7] antiischemia/reperfusion,[8,9] protective agent for transplantation[10,11] and diabetes.[12,13] It, however, has some drawbacks due to its proteinacious character. First, it has very short circulating half-life due to rapid glomerular filtration following I.V. injection.[14] Secondly, SOD can not pass through cell membrane because of the large molecular size and its anionic character.[15] Finally, instability, potential immunogenicity and short supplies are other factors associated with its protein nature. Some of these problems can be circumvented to some degree by special techniques such as liposome-entrapment,[16] scrape-loading[17] and conjugation with polymer.[18,19]

Various shortcomings intrinsic to SOD coupled with the fact that Cu, Fe and Mn exist at the active sites of SOD[20,21] have led to the screening and synthesizing of low molecular weight metal complexes with SOD mimetic activity. Sorenson[22] has reported pharmacological activities, mechanisms of action and physiological applications of copper complexes in which SOD activity of copper complexes is likely to be involved. Ample

evidence has been accumulated in the literature that copper complexes have a variety of effects in biological systems.[23] A large number of investigators have reported the potential use of copper complexes as antiinflammatory,[24-26] anticarcinogenic,[27,28] anticonvulsant,[29] analgesic,[30] antiulcer,[31] antiarthritic/antirheumatic,[32,33] antineoplastic,[34] antidiabetic,[35] antimutagenic[36] and radioprotectant.[37] These apparently diverse effects, at least in part, are likely to be related to the SOD mimeic activity of copper complexes. The area of clinical and experimental applications of copper complexes have shown a degree of similarity with those of SOD, probably because copper complexes have a variety of effects in which copper-dependent enzymes take an important part.[38] The two main functions of copper complexes suggested by Sorenson[39] are 1) copper complexes facilitate transportation of copper to copper-dependent enzymes including SOD. 2) copper complexes mainfest their activity via their own chemical reactivities such as superoxide dismutation. In either case it is worthwhile using copper complexes to substitute for native SOD. Many attempts have been made to synthesize catalytically active chelates with the transition metal ion which disproportionate superoxide anion in a fashion similar to SOD.[40,41] A large number of copper-containing compounds such as copper-salicylates,[42] copper-amino acids,[43,44] copper-oligopeptides,[45,46] as well as iron-porphyrin complexes[47] are found to be effective in scavenging $O_2 \cdot^-$. SOD mimetic activity of various copper-histidine-containing dipeptide complexes was also investigated by pulse radiolysis technique.[48] Several studies have been performed to prepare efficient SOD mimetics on the basis of structure difference to prepare efficient SOD mimetics on the basis of structure difference of chelating ligand.[49] SOD mimetic activity of copper(II) and nickel(II) complexes of macrocyclic polycyclic polyamine derivatives was shown to be dependent on their structure and metal ion. Kimura[50] *et al.* chemically modified structure of a series of macrocyclic complexes searching for more efficient and useful catalyst for superoxide dismutation. Not much work has been done on systematic design and synthesis of copper complexes based on physicochemical properties of ligands. Bijiloo[51] *et al.* have recently reported that SOD activity of a number of copper complexes of substituted 1,10-phenanthrolines is related to steric and field effects of the substituents. Among those complexes mentioned above, particularly copper chelates display marked scavenging effects on superoxide anion at physiological pH values and have been extensively studied as potential SOD mimetics.[52,53]

We prepared copper complexes of a series of SA analogs and measured their SOD activities in order to establish relationships between physicochemical properties of copper complexes of SA analogs and their SOD mimetic activities. The elucidation of the relationship of physicochemical properties of copper complexes to SOD activity may aid in the better understanding of the biochemical basis for the SOD mimetic reaction mechanism of copper complexes as well as in desigining more suitable mimetics.

2. MATERIALS AND METHODS

2-1. Materials

3-Methyl salicylic acid (3-MeSA) and 5-methylsalicylic acid (5-MeSA) were obtained commercially and purified by several recrystallizations from alcohol and water mixture (1/1) and air dried *in vacuo*. SA was recrystallized from methyl alcohol and air dried *in vacuo*. Commercially available 3,5-diisopropylsalicylic acid (DIPS) was obtained as a tan solid and purified by extracting an aqueous solution of its sodium salt, formed with sodium bicarbonate, three times with benzene. The aqueous layer was then acidified with concentrated hydrochloric acid to

obtain the light tan DIPS. All the chemicals mentioned above as well as hydrochloric acid (volumetric standard, 1.0 N), potassium hydroxide (volumetric standard, 0.1 N), sulfuric acid, 3,5-di-*tert*-butylsalicylic acid (DTBS) (zinc salt), ninhydrin, acetic acid, *tert*-butylalcohol, 4-methylaniline, 2-ethylphenol, 4-ethylphenol, 2-isopropylphenol, 4-isopropylphenol, 4-*tert*-butylphenol, 2,4-dimethylphenol, cupric chloride, cytrochrome c from horse heart muscle, brilliant blue G, acetaldehyde. Triton X-100 and allopurinol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methyl alcohol and ethyl alcohol were HPLC grade and were used as supplied by Fisher Scientific Co. (Fair Lawn, NJ). Sodium monophosphate, sodium diphosphate, sodium hydroxide and potassium chloride, all reagent grade, were also from Fisher. Xanthine oxidase (EC 1.1.3.2) from buttermilk, grade I, No. X-1875, suspension in 2.3 M ammonium sulfate, 13.2 mg protein/m*l*, activity 0.59 unit/mg protein, xanthine, bovine albumin, No. A 4378 crystallized and lyophilized, SOD from bovine erythrocytes, lyophilized powder, 98% protein. 3570 unit/mg protein were obtained from Sigma Chem. Co. (St. Louis, MO). Catalase was from Boehringer Mannheim Biochemical Co. (Indianapolis, IN) and Scintiverse Bio-HP was from Fisher. D-glucose[1-^{14}C] was from ICN Biomedicals, INC (Costa Mesa, CA). All other chemicals were also of analytical reagent grade.

2-2. Synthesis of SA Analogs from Various Phenols

SA analogs such as 3-ethylsalicylic acid (3-EtSA), 3-isopropylsalicylic acid (3-ProSA), 3-*tert*-butylsalicylic acid (3-BuSA), 5-ethylsalicylic acid (5-EtSA), 5-isopropylsalicylic acid (5-ProSA) and 3,5-dimethylsalicylic acid (DMSA) were synthesized according to the procedure of Schmitt *et al.*[54]

2-3. Preparation of 3,5-di-*tert*-Butylsalicylic Acid (DTBS)

The commercially available $Zn(DTBS)_2$ was boiled with concentrated HCl. DTBS was then precipitated out, collected and washed with water. It was recrystallized several times from methyl alcohol.

2-4. Peparation of 5-*tert*-Butylsalicylic Acid (5-BuSA)

5-BuSA was prepared according to Foye and Turcotte.[55] Briefly, 20 g of SA, 25 m*l* of *tert*-butyl alcohol and 540 m*l* of 80% sulfuric acid were stirred, during which time the temperature was heated to 75℃ and maintainedd for 1 hr. The resulting solution was allowed to cool overnight, charcoal was added and the mixture was boiled for 5 min. It was filtered and allowed to cool, and the white, crystalline product was isolated and recrystallized from dilute acetic acid.

2-5. Preparation of Copper Complexes

SA analog copper complexes were prepared according to Sorenson[58] with minor modifications. Briefly, 0.025 mole of SA analog was dissolved in 100 m*l* of water with a solution of NaOH (50%), filtered and back-titrated if necessary with a solution of HCl (10%) until pH meter shows the solution to be weakly basic. This solution was dropped into 100 m*l* of water containing 0.05 mole of $CuCl_2$. The resulting green to blue precipitate was removed by filtration and dissolved again in 50 m*l* of boiling water. Recooling resulted in renewed crystallization of the complex. The complex was washed with cold water and then dried at 50~80℃ and 15 mmHg vacuum for 3 days prior to submission to elemental analysis.

Melting points were determined on a Thomas-Hoover apparatus by packing standard capillaries and are uncorrected. The proton NMR spectra were determined on Varian EM 390 spectrometer in deuterochloroform. Chemical shifts are expressed in ppm downfield from inter TMS (tetramethylsilane). Infrared spectra of solids were on Beckman AccuLab TM 4 spectrophotometer as potassium bromide pellets in the 600~4000 cm^{-1} region. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA.

2-6. Protein Determination

Protein content of the EGs was determined according to the method of Bradford[57] using BSA as the calibraton standard.

2-7. Determination of SOD Mimetic Activity of Copper Complexes

The ferricytochome c reduction assay was performed according to Crapo[53] *et al.* with slight modification. Our standard assay was as follows: cytochrome c solution (0.05 mM) and xanthine solution (0.5 mM) were prepared in 0.05 M potassium phosphate buffer solution (PBS, pH 7.4). XOD (stock enzyme) was diluted with the same PBS so that a 10 μl sample would catalyze an absorbance change of 0.025 per min at 550 nm in the absence of copper complex. Cytochrome c solution (0.6 ml), xanthine solution (0.6 ml) and 1.8 ml of the PBS were placed in a 5 ml cuvette with a light path of 1.0 cm, in a thermostated cell compartment at 25℃. Solutions to be assayed were saturated with oxygen by bubbling the gas through the solutions for 30 min. The final reaction mixture contained 10 μM ferricytochrome c and 0.1 mM xanthine. Reactions were initiated by adding 10 μl of XOD (0.008 U) and a stirrer was employed. In the initial assay, the course of the reaction was followed by the increase in absorbance at 550 nm as ferricytochrome c was converted to ferrocytochrome c. The rate of increase in absorbance in the absence of copper complexes was taken as the value 100% activity. Other reaction mixture were then prepared in which copper complex solutions at varying concentrations were added and the resulting mixtures were allowed to be stirred at 25℃ for 5 min. The rate was again monitored after adding XOD to generate $O\cdot^{-}$. The rate of increase in absorbance (ΔA_{550}/min) in the presence of different amounts of copper complexes was calculated in percent of the rate of increase in absorbance in the absence of copper complexes and plotted against the negative logarithm of the copper concentration. The plots obtained were linear over the experimental concentration range studies allowing a determination of EC_{50}, the concentration of copper complex required to inhibit the rate of reduction of ferricytochrome c by 50% under these specified conditions. Inhibition of the reduction of ferricytochrome c by native SOD was used for comparative purposes. Each determination was performed in triplicate.

2-8. Potentiometric Measurement for Stability Constants

Stability constants of copper complexes were measured potentiometrically based on alkaline titration of each ligand solution in the presence of copper ion and listed in Table II. Carbon dioxide was excluded by bubbling with nitrogen gas. Titration was performed by standard CO_2-free 0.1 N KOH traced by Corning pH/ion analyzer 250 (Corning, New York). To obtain useful stoichiometric quantities directly from potentiometric measurements, the pH scale of $[H^+]$ ion concentration rather than (H^+) ion activity was used. The mean ionic activity coefficient, $f_{\pm}$, was need for the conversion of hydrogen ion activity to concentration ($[H^+]=(H^+)/f_{\pm}$). Value of 0.81564 for $f_{\pm}$ was calculated from the Davies equation[59] when I = 0.1 and 13.9965[60] was taken for pK_w of water at 25℃. Numerical evaluation of all the potentiometric data was carried out with the computer program SCOGS2[61] on VAX.

2-9. Preparation of Erythrocyte Ghosts

Fresh blood was withdrawn from a healthy volunteer into vacutainer (Bection Dickinson Vacutainer Systems, Rutherford, NJ) and used within 4 hr of collection. Resealed ghosts were prepared with the method of Steck.[62,63] The preghosts suspended in 0.5 mM PBS (pH 8.0) were incubated with D-glucose[1-^{14}C] (1 μCi) and XOD (1 U) for 15 hr at 4℃ with rotation. The preghosts equilibrated with enzymes and radioactive glucose were scaled by the shear stress inherent in 5-fold passage through a No. 27 gauge hypodermic needle. The EGs were washed with pH 7.4 PBS to

Table I—Salicylic Acid Analogs

Salicylic acid analogs	Molecular formula[a]	mp. ℃	^{1}H-NMR ($CDCl_3$/TMS$_{int}$) δ [ppm], J[Hz]
3-EtSA	$C_9H_{10}O_3$ (166.18)	155-116	1.24(t, J=7.5, 3H); 2.70(q, J=7.5, 2H); 6.86(t, J=7.71, 1H); 7.39(d, J=7.3, 1H); 7.79(d, J=8.0, 1H)
3-ProSA	$C_{10}H_{12}O_3$ (180.20)	74	1.25(t, J=6.9, 6H); 3.34(t, J=7.7, 1H); 6.82(h, J=6.9, 1H); 7.41(d, J=7.3, 1H); 7.73(d, J=8.0, 1H)
3-BuSA	$C_{11}H_{14}O_3$ (194.23)	161-162	1.42(s, t-C_4H_9); 6.85(t, J=7.8, 1H); 7.52(d, J=7.7, 1H); 7.82 (d, J=8.0, 1H)
5-EtSA	$C_9H_{10}O_3$ (166.18)	117-118	1.21(t, J=7.6, 3H); 2.60(q, J=5.7, 2H); 6.93 (d, J=8.5, 1H); 7.36(d, J=8.5, 1H); 7.72(s, 1H)
5-ProSA	$C_{10}H_{12}O_3$ (180.20)	124-125	1.23(d, J=6.9, 6H); 2.87(h, J=6.9, 1H); 6.94(d, J=8.6, 1H); 7.40(d, J=8.6, 1H); 7.76(s, 1H)
5-BuSA	$C_{11}H_{14}O_3$ (194.23)	153	1.30(s, t-C_4H_9); 6.94(d, J=8.8, 1H); 7.57(d, J=5.1, 1H); 7.89(s, 1H)
DMSA	$C_9H_{10}O_3$ (166.18)	187-188	1.32(s, 3H); 1.44(s, 3H); 7.60(s, 1H); 7.82(s, 1H)
DTBS	$C_{15}C_{22}O_3$ (250.34)	168	2.23(s, t-C_4H_9); 2.26(s, t-C_4H_9); 7.20(s, 1H); 7.55(s, 1H)

[a] The microanalyses were in satisfactory agreement with the calculated values within ± 0.3%

Table II—Stability Constants of Copper(II)-Salicylic Acid Analogs[a]

Ligand	Log k_1[b]	Log k_2[b]	Log β_2[c]
SA	10.53	7.89	18.42
3-MeSA	10.66	6.46	17.12
3-EtSA	10.62	6.48	17.10
3-ProSA	10.69	6.53	17.22
3-BuSA	10.75	6.60	17.35
5-MeSA	10.80	8.32	19.12
5-EtSA	10.84	8.36	19.20
5-ProSA	10.83	8.43	19.26
5-BuSA	10.86	8.47	19.33
DMSA	10.74	7.09	17.83
DIPS	10.78	7.20	17.98
DTBS	10.89	7.23	18.12

[a] Ionic strength was adjusted to 0.1 M with KCl. SCOG2 program was run to estimate stability constants. [b] k_n=K (CuL+L=CuL). [c] β_n=K(Cu+nL=CuLn).

remove unincorporated XOD and radioactive glucose.

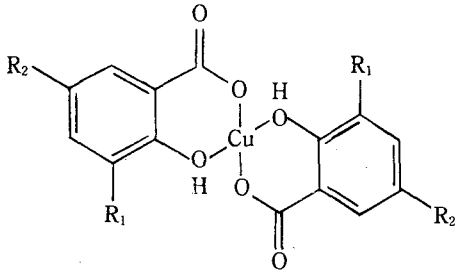
2-10. Incubation of EGs in the Presence of Copper Complexes

EGs were pretreated with varying concentration of copper complexes for 3 hr prior to conducting experiments. 5 m*l* of these pretreated EGs were put in 25 m*l* Erlenmeyer flasks in air. Reaction was initiated by adding 20 mM acetaldehyde. Reaction mixture was shaken gently while being incubated at 25℃ for 5 hr. At specified time intervals, 300 μ*l* of the reaction mixture were taken out for scintillation counting.

2-11. Liquid Scintillation Counting

D-glucose[1-^{14}C] sealed into the EGs along with XOD was released as XOD/acetaldehyde system attacks the EGs, and lysis was measured in terms of its release into the medium. Supernatant fractions (0.1 m*l*) after terminating the reaction by adding allopurinol[64] (57 μM) to inhibit the activity of XOD were obtained and counted by scintillation methods in 10 m*l* of Scintiverse Bio-HP cocktail with a Beckman LS1701 liquid scintillation system. The total radioactivity was measured by counting 100 μ*l* of the whole suspensions of EGs in 10 m*l* of cocktail and experimental results were expressed as percentage of the total radioactivity.

2-12. SAR Study



R_1	R_2	R_1	R_2
H	H	methyl	H
H	methyl	ethyl	H
H	ethyl	i-propyl	H
H	i-propyl	t-butyl	H
H	t-butyl	methyl	methyl
i-propyl	i-propyl	t-butyl	t-butyl

Figure 1—Copper complexes of salicylic acid analogs.

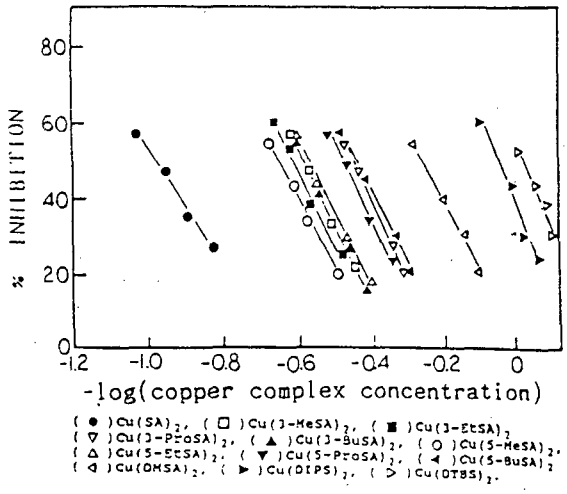


Figure 2—Estimation of EC_{50} for copper complex.

The physicochemical model for biological activity in this investigation assumes that SOD mimetic activities of copper complexes are governed mainly by electronic parameter, steric parameter, and hydrophobic parameter.[65] The Hansch-Fujita π[66] constant reflects lipophilic character of the substituent. E_s is Taft's steric effect[67] and σ is the substituent electronic effect of Hammett.[68] The relative importance of these three parameters was evaluated, which might provide information on the design of efficient SOD mimetics and the mechanism of action of copper complexes. A general equation[69] for the multiparameter approach to structure-activity relationships (SAR) in drug is:

$$-\log C = k_1(\sum\pi)^2 + k_2(\sum\pi) + k_3(\sum\sigma) + k_4(E_s) + k_5$$

where the concentration of drug required to produce a specified standard response is correlated with the change in π, σ and E_s caused by structural modifications within a class. Statistical comparisons of SAR using the Hansch procedure[70] were performed on a IBM 3084 computer employing the SAS program.

3. RESULTS AND DISCUSSION

All the elementary analyses, NMR spectra and IR spectra were in good agreement with the proposed formulae. The IR spectra of all the SA analogs have strong band in common at 1650~1655 cm^{-1} which corresponds to COOH stretching. Fig. 1 shows the structure of copper complexes prepared.

Cytochrome c is reduced by superoxide radicals generated by the action of XOD on xanthine. SOD catalyzes the dismutation of superoxide radicals in buffer solutions as shown by inhibition of cytochrome c reduction. Plotting % inhibition against negative logarithm of copper complex concentration (Fig. 2) showed straight line from which EC_{50} value was then determined by interpolation. Table III lists EC_{50} value for each copper complex. The EC_{50} value for native SOD was 4.49 nM and that of $Cu(SA)_2$ was 9.76 μM. However comparing the molecular weight of SOD (32 kDa) and that of $Cu(SA)_2$ (355.8), copper complexes can function as effectively as SOD on weight per weight basis.

As shown in Fig. 3, in contrast to native SOD, all the copper complexes tested showed a similar pattern of losing their SOD mimetic activities in the presence of BSA around 10 g/*l*, even less than the physiological concentration 40 g/*l*. This result indicated that even though copper complexes showed efficient

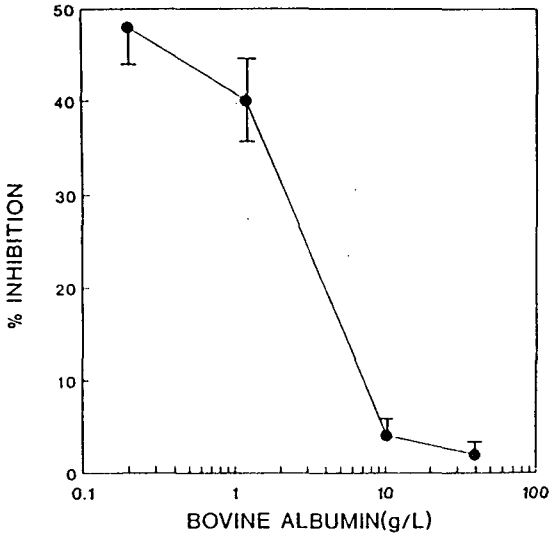


Figure 3—SOD activity of copper complex in the presence of BSA. Points, means of activities of 12 copper complexes; vertical bars, range. EC_{50} value listed in Table III was used for each copper complex.

Table III—Superoxide Dismutase Mimetic Activity and Physiochemical Data for Copper(II)-Salicylic Acid Analogs[a]

Copper complex	EC_{50}[b] (μM)	E_g[c]	$\sum\sigma$[d]
$Cu(SA)_2$	9.76	0.00	0.00
$Cu(3\text{-}MeSA)_2$	3.91	−1.24	−0.07
$Cu(3\text{-}EtSA)_2$	4.12	−1.31	−0.07
$Cu(3\text{-}ProSA)_2$	2.87	−1.71	−0.07
$Cu(3\text{-}BuSA)_2$	3.91	−2.78	−0.10
$Cu(5\text{-}MeSA)_2$	4.47	−1.24	−0.17
$Cu(5\text{-}EtSA)_2$	3.71	−1.31	−0.15
$Cu(5\text{-}ProSA)_2$	3.18	−1.71	−0.15
$Cu(5\text{-}BuSA)_2$	2.76	−2.78	−0.20
$Cu(DMSA)_2$	1.83	−2.48	−0.24
$Cu(DIPS)_2$	1.10	−3.42	−0.24
$Cu(DTBS)_2$	0.94	−5.56	−0.30

[a]SOD mimetic activity was measured in pH 7.4 potassium phosphate buffer at 25℃. [b]Concentration of copper complex to inhibit cytochrome c reduction assay by 50%, [c]Adapted from ref. 83. [d]Adapted from ref. 82, and calculated with respect to the carboxyl group.

SOD mimetic activity *in vitro*, it may not survive chelating biomolecules such as serum albumin *in vivo*. However the exact nature of active therapeutic components after they enter in biological systems still remains in doubt. This result was consistent with the observation of Miesel *et al.*[71]

One of the most investigated mechanisms for inducing tissue damage by free radical is lipid peroxidation.[72,73] Alterations in membrane fluidity of human erythrocyte can be induced by direct lipid peroxidation[74] in which free radicals play an important role. Free radicals generated in aqueous phase have been shown to destory erythrocyte membranes. This results in oxidation of unsaturated lipids and proteins, and ultimately cause hemolysis through disruption of permeability barriers. The oxidation of erythrocyte membranes serves as a model for the peroxidative damage to biomembranes mediated by a free radical chain mechanism by molecular oxygen.[75] Niki *et al.*[76] have observed that free radicals can attack erythrocyte membranes to induce the chain oxidation of lipids and proteins leading to hemolysis. These membrane damage have been shown to be inhibited by free radical scavengers.[76,77] Membrane damaging effects of $O_2\cdot^-$ coupled with the fact that SOD mimetics can scavenge $O_2\cdot^-$ led us to investigate the protective effect of SOD mimetics against $O_2\cdot^-$ employing human erythrocyte ghost as a membrane model. XOD enclosed within EGs generated superoxide anion as it catalyzed the oxidation of acetaldehyde, its substrate, entering from the suspending medium.[78] The lytic effects of the XOD-acetaldehyde reaction were diminished by copper complexes which might be either transported into EGs or reside in the membrane to show protection against oxidative attack. SOD showed no protective effect probably because it cannot get into the site of deleterious oxygen radical generation. We measured % release at 3 hr in the presence of varying concentration of copper complexes. Plotting % release of D-glucose[1-^{14}C] against logarithm of concentration produced a straight line suggesting that copper complexes

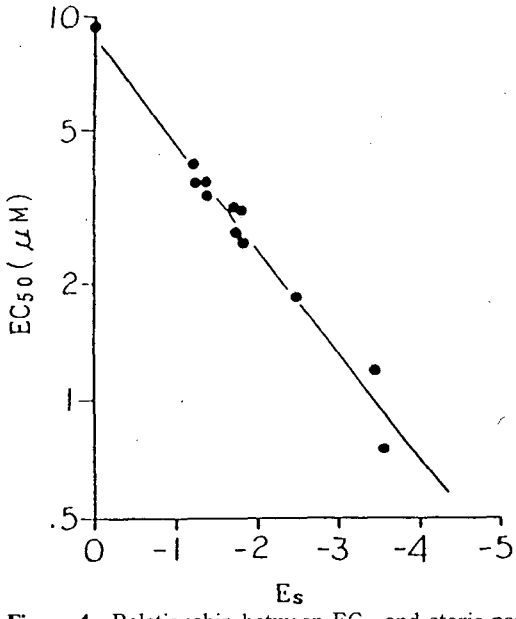


Figure 4—Relationship between EC_{50} and steric parameter.

inhibited lysis of EGs in a concentraion dependent manner under our experimental conditions. We estimated the concentration of copper complex which produces the D-glucose[1-^{14}C] release by 50% (IC_{50}) from the above plot. Fig. 4 shows these plots and the IC_{50} value for each copper complex obtained from this graph is listed in Table IV.

The equations (eq.) obtained by correlating the SOD mimetic activity with the physicochemical parameters for the copper complexes are shown in Table IV. Eq. (1) in Table IV was derived using only $\sum\sigma$ as the independent variable. Eq. (2) derived only employing E_s showed a good correlation. However, addition of the $\sum\sigma$ into Eq. (2) did not improve the correlation, but slightly increased the standard deviation (S.D.) with concomitant decrease in adjusted-r^2. This was not statistically important. Comparing the statistics for Eq. (2) and (3), it may be concluded that E_s plays a key role in the SOD mimetic activity of copper complexes in the XOD/xanthine system. James and Williams[79] reported the oxidation-reduction potentials of some copper complexes. They showed that the chelating ligand affects the redox potential of cupric and cuprous complex couple. In light of this, Bijiloo *et al.*[51] suggested that the effect of E_s may be explained in terms of alteration in redox potential of Cu(I) complex/Cu(II) complex. Considering these together, further experiments on redox potential of copper complexes are required for better understanding of the relative role of E_s in SOD mimetic activity. Fig. 4 shows a linear relationship between the SOD mimetic activity of copper complexes and E_s. As the SOD mimetic activity involves a redox cycle of Cu (II) and Cu(I) it is to be expected that redox potential of a copper(II) complex/copper(I) complex exerts influences on the SOD activity. Redox potential appears to depend on their ligands. Electron factors and steric factors which reflect changes in the acid dissociation constants, and consequent changes in stability constants may influence the SOD activity.

In biological systems passage across a series of hydrophilic-lipophilic barriers is responsible for the transport of a drug or a

Table IV—Equations Correlating the Superoxide Dismutase Mimetic Activity with Physicochemical Data for Copper(II)-Salicylic Acid Analogs[a]

Equation	Adjusted-r^2	S.D.	F ratio
(1) $-\log EC_{50} = -2.73(\pm 1.076)^b\sum\sigma - 0.87(\pm 0.182)$	0.7382	0.3253	32.018
(2) $-\log EC_{50} = -0.28(\pm 0.031)E_s - 0.97(\pm 0.063)$	0.9729	0.1047	395.47
(3) $-\log EC_{50} = -0.26(\pm 0.064)E_s - 0.25(\pm 0.709)\sum\sigma - 0.98(\pm 0.065)$	0.9718	0.1067	190.735

[a] Statistical analyses were performed with the SAS program, [b] Values in parentheses are the 95% confidence intervals.

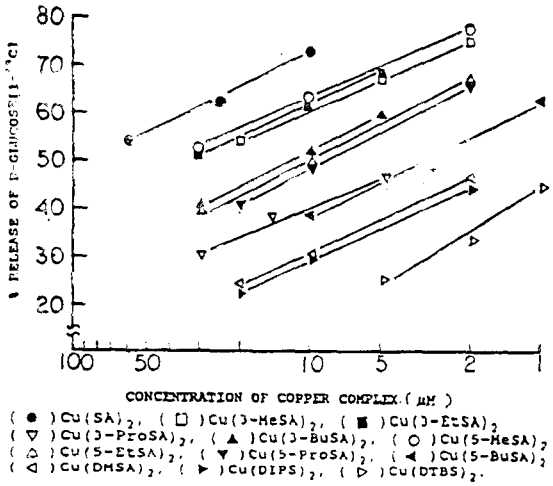


Figure 5—Estimation of IC_{50} values for copper complexes.

bioactive molecule from the site of administration or biosynthesis to the target tissues. In this context, it is likely that the lipophilic character of copper complexes may play a major role in SOD mimetic activity in the membrane model system using EGs. We prepared a superoxide generating systen within the EGs, then investigated the superoxide scavenging effect of copper complexes. To be an effective scavenger, copper complexes should enter EGs by a passive transport mechanism, which depends mainly on the lipophilicity of copper complexes.

Table V—Protective Effects of Copper Complexes against D-Glucose [1-^{14}C] Release from Erythrocyte Ghosts and Their Physicochemical Properties

Copper complex	IC_{50} (μM)	[a]E_s	[b]$\sum\sigma$	[c]$\sum\pi$
$Cu(SA)_2$	84.56	0.00	0.00	0.00
$Cu(3\text{-}MeSA)_2$	38.12	−1.24	−0.07	0.50
$Cu(3\text{-}EtSA)_2$	13.10	−1.31	−0.07	1.00
$Cu(3\text{-}ProSA)_2$	3.12	−1.71	−0.07	1.30
$Cu(3\text{-}BuSA)_2$	1.19	−2.75	−0.10	1.60
$Cu(5\text{-}MeSA)_2$	31.44	−1.24	−0.17	0.50
$Cu(5\text{-}EtSA)_2$	8.41	−1.31	−0.15	1.00
$Cu(5\text{-}ProSA)_2$	4.83	−1.71	−0.15	1.30
$Cu(5\text{-}BuSA)_2$	1.75	−2.78	−0.20	1.68
$Cu(DMSA)_2$	10.57	−2.48	−0.24	1.00
$Cu(DIPS)_2$	0.64	−3.42	−0.24	2.60
$Cu(DTBS)_2$	3.23	−5.56	−0.30	3.36

[a]Adapted from ref. 83
[b]Adapted from ref. 82 and calculated with respect to the carboxyl group.
[c]The Hansch-Fujita π constant was estimated by the additive principle of Lien *et al.*[80,81]

The linear combination of the three parameters, $\sum\pi$, E_s and $\sum\sigma$, did not give a signi-

Table VI—Equations Correlating Protective Effect to Their Physigochemical Properties[a]

Equation	Adjusted-r^2	S.D.	F ratio
(1) $-\log IC_{50} = 0.00(\pm 0.234)^b\sum\pi - 1.56(\pm 0.469)$	0.6018	0.9336	17.024
(2) $-\log IC_{50} = 1.07(\pm 0.958)\sum\pi + 0.35(\pm 0.625)E_s - 1.49(\pm 0.476)$	0.6254	0.9056	10.181
(3) $-\log IC_{50} = 0.40(\pm 0.460)\sum\pi + 1.22(\pm 4.829)\sum\sigma - 1.49(\pm 0.555)$	0.5731	0.9667	8.383
(4) $-\log IC_{50} = 1.09(\pm 1.071)\sum\pi - 0.38(\pm 0.824)E_s - 0.35(\pm 5.962)E_s - 1.50(\pm 0.564)$	0.5795	0.9594	6.053
(5) $-\log IC_{50} = 1.32(\pm 0.426)(\sum\pi)^2 + 1.87(\pm 0.677)\sum\pi + 0.11(\pm 0.454)E_s - 0.75(\pm 3.189)\sum\sigma - 2.13(\pm 0.426)$	0.8880	0.4956	22.813
(6) $-\log IC_{50} = 0.32(\pm 0.146)(\sum\pi)^2 + 1.88(\pm 0.635)\sum\pi - 0.20(0.344)E_s - 2.14(\pm 0.394)$	0.8977	0.4731	33.186
(7) $-\log IC_{50} = 0.33(\pm 0.142)(\sum\pi)^2 + 1.77(\pm 0.535)\sum\pi + 1.22(\pm 2.423)\sum\sigma - 2.15(\pm 0.393)$	0.8971	0.4745	32.979
(8) $-\log IC_{50} = 0.33(\pm 0.142)(\sum\pi)^2 + 1.69(\pm 0.505)\sum\pi - 2.21(\pm 0.371)$	0.8831	0.4837	46.968

[a]Statistical analyses were performed with the SAS program.
[b]Values in parentheses are the 95% confidence intervals.

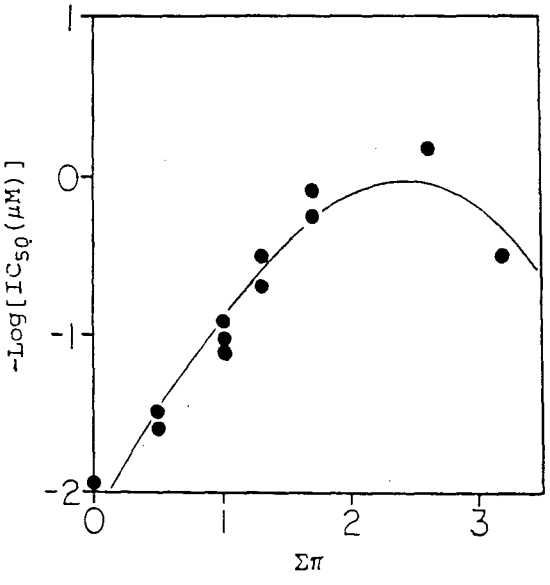


Figure 6—Parabolic relationship between $-\log[IC_{50}]$ and $\sum\pi$.

ficant improvement, with adjusted-r^2 values less than 0.63 (Table VI). However, addition of the $(\sum\pi)^2$ markedly increased adjusted-r^2 suggesting that a significant improvement is obtained with a parabolic equation. We also found electronic effects to be clearly unimportant since no significant improvement was obtained by addition of this term. However, the addition of the E_s term of Eq. (8) in Table VI resulted in slightly better correlation as shown by Eq. (6). Judging from the statistics in Table VI, Eq. (6) appears to be the best fit equation with a parabolic dependent on $\sum\pi$. This observation is consistent with the assumption that hydrophobic properties of copper complexes play a prominent role in penetrating into the EGs. It can be said from the above results that the ability of copper complexes for scavenging superoxide anion, as measured by protection of EGs from oxidative attack, can be reasonably predicted from structural features such as lipid solubility.

In summary, hydrophobicity appears to be the most important factor, whereas steric factor seems to be of secondary importance and electronic factor is of little importance.

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