Bis(3,5-diisopropylsalicylato)copper(II), a Potent Radioprotectant with Superoxide Dismutase Mimetic Activity

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Superoxide disproportionation may partially account for the noteworthy radioprotectant activity of bis(3,5-diisopropylsalicylato)copper(II) [$Cu^{II}(3,5\text{-dips})_2$]. Groups of mice treated with $Cu^{II}(3,5\text{-dips})_2$ 3 or 24 h before exposure to a lethal dose of γ-radiation had survival rates of 33% and 58%, respectively. These results suggest that copper complexes might be developed for protection of normal tissues in association with cancer radiotherapy and protection against occupational exposures to hazardous radiation.

It is now understood that oxygenated aqueous solutions exposed to high energy γ- or X-rays yield radiolytic products according to the following reaction:[1]

$$H_2O \text{ -wvy-} \rightarrow e^- + H\cdot + H_2 + HO\cdot + H_3O^+ + H_2O_2$$

Energy-rich radicals H· and e^- lead to superoxide formation at diffusion-controlled rates ($>10^{10}$ M^{-1} s^{-1}) in the presence of triplet-state dioxygen according to the following reactions:[2-5]

$$H\cdot + \cdot O\text{–}O\cdot \rightarrow HO\text{–}O\cdot \rightarrow H^+ + {}^-O\text{–}O\cdot \qquad \text{pH 4.7}$$

$$e^- + \cdot O\text{–}O\cdot \rightarrow {}^-O\text{–}O\cdot$$

Formation of superoxide partially accounts for the well-known oxygen enhancement of radiation-induced cell damage.

The copper-dependent and zinc-modulated superoxide dismutase (Cu–Zn SOD) normally found in cells of all aerobic organisms has a recognized protective role in scavenging superoxide:[6,7]

$$2\,{}^-O\text{–}O\cdot + 2H^+ \rightarrow H_2O_2 + \cdot O\text{–}O\cdot$$

The rate of this reaction is also diffusion controlled, $k = 1.3 \times 10^9$ M^{-1} s^{-1} (3).

The specificity with which Cu–Zn SOD catalyses the destruction of superoxide together with the demonstrated radioprotection of enzymes, bacteriophage, bacteria, mycoplasma, and mammalian cells with Cu–Zn SOD prompted Petkau and his colleagues[8-10] to examine the prophylactic effect of Cu–Zn SOD on survival of whole-body 6.5 Gy (1 Gy = 100 rads) X-irradiated mice. The maximal effective dose of Cu–Zn SOD was 1.1 μM/kg; higher and lower doses were less effective when the enzyme was given intravenously (iv) 1 h prior to irradiation, the time at which maximum concentrations of ^{125}I-labeled enzyme were found in bone marrow and bone marrow stem cells. Pretreatment with 1.1 μM/kg of Cu–Zn SOD increased the $LD_{50/30}$ dose of radiation from 6.3 to 7.0 Gy and 10% survival was observed with a $LD_{100/30}$ dose of radiation, 8.2 Gy. The same dose of Cu–Zn SOD given 1 h before and 1 h after irradiation further increased the $LD_{50/30}$ dose to 8.7 Gy and 17% of the mice survived when the radiation dose was increased to 10 Gy.

Table I. SOD-Like Activity, Percent Reactivity, and Rate of Superoxide Dismutation for Some Copper Salicylates and Cu–Zn SOD Obtained with the Xanthine–Xanthine Oxidase Superoxide Generating System[a]

complex	concn (μM) of compd[d]	% reactivity	rate, $\times 10^9$ m^{-1} s^{-1}
Cu–Zn SOD	0.02	100	1.3[b]
$Cu^{II}(3,5\text{-dips})_2$	2.9	0.70	1–2[c]
$Cu^{II}(salicylate)_2$	4.6	0.65	1.6[b]

[a] Reference 11. [b] Reference 13. [c] Reference 14. [d] Concentration required for 50% inhibition of superoxide reduction of nitroblue tetrazolium.

Since it was known that $Cu^{II}(3,5\text{-dips})_2$ also catalyzes the disproportionation of superoxide[11] and that it is a lipid-soluble complex (soluble in diethyl ether) capable of crossing lipid membrane barriers, it was selected for evaluation as a radioprotectant.

Results and Discussion

Results presented in Figure 1 show that $Cu^{II}(3,5\text{-dips})_2$ is a remarkably effective radioprotectant. Thirty-three percent (eight mice) of the mice treated 3 h before irradiation survived and none died after day 18 postirradiation. Fifty-eight percent (14 mice) of the mice treated 24 h before irradiation survived and none died after day 19 postirradiation. All of the control mice died by day 18 postirradiation. When similarly treated mice were irradiated with 12 Gy, there were no survivors.

The radioprotection observed with the 10 Gy dose does support the need to study other lipid-soluble copper complexes as radioprotectants. While conditions under which data were obtained with $Cu^{II}(3,5\text{-dips})_2$ were different from those used by Petkau and his colleagues to determine the radioprotectant activity of Cu–Zn SOD, a comparison of these data suggest that $Cu^{II}(3,5\text{-dips})_2$ has the potential of being much more effective than Cu–Zn SOD. $Cu^{II}(3,5\text{-dips})_2$ produced much greater protection, 33% or 58% survival depending on the pretreatment interval, than Cu–Zn SOD.

Greater protection with $Cu^{II}(3,5\text{-dips})_2$ may not be due to superoxide disproportionation alone. Data presented in Table I show that $Cu^{II}(3,5\text{-dips})_2$ is not as efficient as Cu–Zn SOD in disproportionating superoxide although the kinetics of disproportionation by copper salicylates suggest equivalent diffusion-controlled disproportionation at the active copper site. Smaller molecular size and lipophilicity of $Cu^{II}(3,5\text{-dips})_2$ may, however, facilitate tissue distribution and cellular membrane transport, which is not possible for Cu–Zn SOD. The large molecular size of Cu–Zn SOD and its anionic character prevent its transport across cell membranes and, as a result, its protective effect can only

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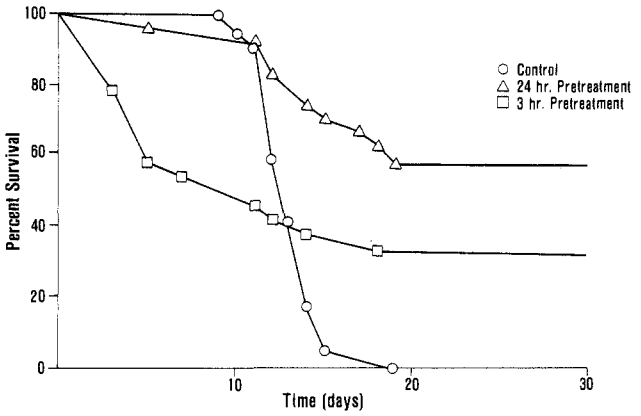

Figure 1. Survival of lethally irradiated mice treated with $Cu^{II}(3,5\text{-dips})_2$.

Table II. Reaction Rates for Cupric Complexes with Hydroxyl Radical, Hydrated High-Energy Electrons, and Hydrogen Atoms[a]

ligand	pH	rate, $\times 10^9$ M^{-1} s^{-1}
$Cu^{II}(L)_2 + HO\cdot \rightarrow Cu^{III}(L)_2 + HO^-$		
H_2O	7.0	0.35
$H_2NCH_2CH_2NH_2$	6.5	3.0–0.6
$H_2NCH_2CO_2$	6.1	1.5–0.3
$CH_3CH(NH_2)CO_2$	6.3	1.4–0.3
$H_2NCH_2CH_2CO_2$	5.8	1.2–0.2
$CH_3CH_2CH(NH_2)CO_2$	6.1	2.0–0.4
$CH_3CH_2CH(NH_2)CH_2CO_2$	6.0	1.2–0.2
$NH_2CH_2CH_2CH_2CO_2$	4.8	1.1–0.2
Na_2EDTA	7.0	4
$Cu^{II}(L)_2 + e^-_{aq} \rightarrow Cu^{I}(L)_2$		
H_2O	7	30
Na_2EDTA	12	10
$Cu^{II}(L)_2 + H\cdot \rightarrow Cu^{I}(L)_2 + H^+$		
H_2O	7	0.06–0.6

[a] Reference 15.

be exerted in the extracellular spaces. In addition, small-molecular-weight copper complexes are known to react with hydroxyl radical, electrons, and hydrogen atoms at rates that are also diffusion controlled, as shown in Table II. Hydroxyl radicals, energetic electrons, and hydrogen atoms would be expected to destroy Cu–Zn SOD by reacting with the protein of the enzyme before it reached the copper-dependent active site. In summary, lipophilicity and reaction with superoxide, hydroxyl radicals, electrons, and hydrogen atoms may account for the greater radioprotectant activity of $Cu^{II}(3,5\text{-dips})_2$.

Effective and less toxic radioprotectants are needed for protection of normal tissues of patients undergoing radiation therapy for neoplastic disease. The most effective radioprotectant developed to date, *S*-[2-[(3-aminopropyl)amino]ethyl]phosphorothioic acid (WR2721), produces a number of undesirable side effects. Ongoing clinical trials have been complicated by the production of emesis, hypertension, hypotension, somnolence, and allergy.[16,17]

Concentrations of plasma copper complexes are known to increase in neoplastic disease states and return to normal with remission.[18] Since copper complexes have antineoplastic,[19-30] anticarcinogenic,[31,32] and antimutagenic[33] activities, the increase in plasma copper complexes in active neoplastic diseases and their decrease in remission have been interpreted as a component of physiologic responses that facilitate remission.[18] The use of copper complexes as radioprotectants has the potential of supporting this physiologic response to neoplastic disease in addition to protecting normal tissues from effects of superoxide and its radical products, produced with ionizing radiation. Development of radioprotectants with anticancer, anticarcinogenic, and antimutagenic activities is a particularly exciting possibility.

Copper complexes also offer protection against doses of ionizing radiation larger than those used to irradiate neoplasms. Accidental exposure to similar and slightly larger doses of radiation produces lethal immunoincompetence due to suppression of bone marrow stem cell division and inflammation (hematopoeitic syndrome).[34,35] Larger doses of irradiation produce immunoincompetence,

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inflammation, and gastiric ulceration (gastrointestinal syndrome).[34,35] Still larger doses produce, in addition to symptoms of hematopoeitic and gastrointestinal syndromes, damage to the central nervous system leading to tremors and convulsions prior to death (CNS syndrome).[34,35] Copper (complexes) have been shown to be required for immunocompetence.[36,37] Many copper complexes, including $Cu^{II}(3,5\text{-dips})_2$, have been shown to have antiinflammatory activity and promote wound healing.[18] They also have antimicrobial activity,[19] antiulcer activity,[18] and anticonvulsant activity.[38,39] These effects are especially desirable in protecting against the hematopoeitic, gastrointestinal, and central nervous system syndromes induced by progressively increasing doses of ionizing radiation.

Conclusion

Since copper is an essential metalloelement, and as such required by all living cells, and appears to have a physiologic role in homeostasis, it may be possible to synthesize effective and nontoxic copper complexes for use in radioprotection. In toto, their pharmacologic effects are especially desirable in radioprotectants required for the protection of normal tissues in cancer patients undergoing radiotherapy and for those individuals who may be at risk with regard to hazardous effects of exposure to higher doses of ionizing radiation.

Experimental Section

Copper 3,5-diisopropylsalicylate was synthesized according to a published procedure.[12] One group of 22 control and two groups of 24 treated 8–10-week-old female B6CBF1 mice (Cumberland View Farms, Clinton, TN) were used. Control animals were given a subcutaneous (sc) injection of 0.3 mL of vehicle (0.25% Tween 80 in 0.9% pyrogen-free sterile saline) 24 h before irradiation. One treatment group was given a single sc injection of 0.49 mM/kg of $Cu^{II}(3,5\text{-dips})_2$ in 0.3 mL of vehicle 3 h before irradiation and the other group was given the same treatment 24 h before irradiation.

Control and treated mice were placed in Plexiglas cages and irradiated bilaterally with a ^{60}Co source at a rate of 0.4 ± 0.004 Gy/min for 25 min to deliver a projected LD 100/30 dose of 10 ± 0.1 Gy. This radiation dose was measured with an electrometer connected to 0.05-mL NBS-calibrated ion chambers positioned inside wax phantom mice placed in Plexiglas cages. The tissue to air ratio for these phantom mice has been determined to be 97%. These mice were then housed five mice/cage and fed mouse chow and water ad libitum for the 30-day observation period.

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Synthesis and Antitumor Activity of Tropolone Derivatives. 1

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Treatment of tropolones with benzaldehyde diethyl acetals gave monotropolone (**12**) and bistropolone (**13**) derivatives at the benzylic position, whereas the related 1-ethoxyisochroman and the diethyl acetals of crotonaldehyde and cinnamaldehyde gave only the monotropolone derivatives (**5**, **10**, or 11). The monotropolone derivatives (**5**, **10**, 11, and **12**) had poor potency against P388 leukemia in mice, but the bistropolone derivatives (**13** and 14) showed significant potency and prolongation of life.

We were interested in the molecular modification of tropolones for the search of new antitumor agents, especially focused on 4-isopropyltropolone (hinokitiol, β-thujaplicin) (**2**), which naturally occurs in the plants of *Chamaecyparis* species.[1]

We found that the reaction of tropolone (**1**) or hinokitiol (**2**) with 1-ethoxyisochroman, considered to be the intramolecular diethyl acetal of benzaldehyde, gives 3-(isochroman-1-yl)- or 3-(isochroman-1-yl)-6-isopropyl tropolone (4 and **5**, respectively).[2] This finding prompted us to study the reactivity of tropolones with acetals.[2] It was found that hinokitiol (**2**) on treatment with benzaldehyde diethyl acetals gives 3-(α-ethoxybenzyl)-6-isopropyltropolones (**12**) and α,α-bis(2-hydroxy-6-isopropyltropon-3-yl)toluenes (**13**). In addition, hinokitiol (**2**) was found to inhibit the growth of KB cells at low concentration (in vitro system) but to be inactive in the survival test of P388 mice (in vivo system)[3] On the other hand, **5** and **13a** were found to be active both in the in vitro and in vivo systems.[3]

These results led us to test for the antitumor activity of hinokitiol derivatives previously prepared[2] and to expand the program to the preparation of compounds related to **5** and **13a**. This paper describes the syntheses of new

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