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ANTICARCINOGENIC REACTIVITY OF COPPER-DISCHIFFBASES WITH SUPEROXIDE DISMUTASE-LIKE ACTIVITY

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$CuPu(Py)_2$ and $CuPu(Im)_2$, two novel dischiffbase coordinated low Mr active centre analogues of Cu_2Zn_2 superoxide dismutase, were shown to effectively catalyze the production of hydroxyl radicals in the presence and absence of TPA activated polymorphonuclear leukocytes. These stable copper chelates exhibited a pronounced anticarcinogenic reactivity in male Sprague Dawley rats implanted with Walker 256 carcinosarcoma cells. When four doses of 5 μmol/kg $CuPu(Py)_2$ and $CuPu(Im)_2$, respectively, were administered intratumorally, reduction in tumor size, delay of metastasis and a significant increase in survival of the hosts were observed, resulting in 75% of total remissions. 60% of the animals recovered totally from the carcinosarcoma, when $CuPu(Py)_2$ was applicated intravenously.

KEY WORDS: Dischiffbase coordinated low Mr copper chelates, SOD mimetic activity of, TPA activated PMNs, lucigenin mediated chemiluminescence in unseparated human blood, Walker 256 carcinosarcoma.

ABBREVIATIONS: SOD Cu_2Zn_2 superoxide dismutase (EC 1.15.1.1); PMN polymorphonuclear leukocyte; TPA 12-o-tetradecanyolphorbol-13-acetate; $CuPu(Py)_2$ {[N,N′-bis(2-pyridylmethylene-1,4-butanediamine](N,N′,N″,N‴)}-copper(II); $CuPu(Im)_2$ {[1,8-di(2-imidazolyl)-2,7-diazaoctadiene-1,7]-(N,N′,N″,N‴)-copper(II); $Cu(Sal)_2$ copper-salicylate, $Cu(Ser)_2$ copper-serinate; Cu-thiocin 1:1 complex of Cu^{2+} with desferrithiocin isolated from Streptomyces antibioticus; CuDIPS copper-(3,5-diisopropyl-salicylate)$_2$; WS 256 Walker 256 carcinosarcoma; cpm counts per minute; TBA 2-thiobarbituric acid; $[I_{50}]$ copper concentration required to inhibit the oxidative burst dependent production of superoxide of activated PMNs by 50%, NBT nitro blue tetrazoliumchloride.

INTRODUCTION

Bioinorganic metal complexes, including Fe-Bleomycin, copper-salicylates, and cis-diaminedichloroplatinum(II) as well as ruthenium-polypyridyls and -phenanthrolines possess pronounced antineoplastic reactivities both *in vivo* and *in vitro*.[1-8] Quite frequently their anticarcinogenic activity is thought to be attributable to 'site-specific' damages of DNA by oxygen free radicals. In the absence of competitive biological chelators, low M_r copper chelates, including the antitumor agent CuDIPS,[3-6] catalyze the dismutation of superoxide to hydrogen peroxide and oxygen substantially faster than cytosolic Cu_2Zn_2 superoxide dismutase.[9] However, none of those acetate type low M_r SOD mimics survive serum albumin with its specific copper binding site of pk 16.2.[10] Thus, their tumoricidal, radioprotectant, bactericidal and antiinflammatory reactivities reported, are quite frequently obscured.[11-14] By way of contrast, the

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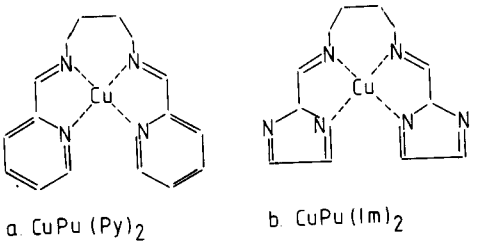

FIGURE 1 Dischiffbase coordinated low M_r copper chelates with superoxide dismutase mimetic activity.

coordination of copper in the dischiffbases involving putrescine and pyridine-2-aldehyde {$Cu(Pu(Py)_2$} or imidazole-2-aldehyde {$CuPu(Im)_2$}, respectively (Figure 1), results in SOD mimics, capable to tolerate serum in their intact form (pk 16.2 and 17.2).[15,16]

The superoxide dismuting reactivities of these low M_r copper chelates are 10 times better compared to that of the acetate type complexes (e.g. CuDIPS), and are virtually identical to that of native Cu_2Zn_2 superoxide dismutase.[17] For example, in the presence of an increased flux of reactive oxygen species, originating from activated polymorphonuclear leukoctyes and macrophages in whole human blood, $CuPu(Py)_2$ and $CuPu(Im)_2$ remain stable and dismute superoxide with similar [I_{50}]-values as Cu_2Zn_2 superoxide dismutase (0.4 μM).[18] Cu^+ is known to effectively catalyze the production of hydroxyl radicals in the presence of H_2O_2, subsequently causing lipid peroxidation, enzyme inactivation and DNA degradation.[19] In this context it was of utmost interest to examine the Fenton-like reactivity of our Cu-dischiffbases in the ·OH-detecting 2-deoxyribose fragmentation assay.[20] Additionally, this assay was modified to conditions coming close to physiological events, by using human PMNs, known to be a suitable source of reactive oxygen species.

Apart from the *in vitro* experiments, the fast growing Walker 256 carcinosarcoma[21,22] transplanted into male Sprague Dawley rats seemed most appropriate to study the anticarcinogenic reactivity of $CuPu(Py)_2$ and $CuPu(Im)_2$ *in vivo*. Parameters, including tumor size, plasma copper concentrations and PMN response were monitored during the time course of carcinogenesis. Special attention was paid to plasma Cu_2Zn_2 superoxide dismutase activities, indicating the necrosis of tumor tissue.

MATERIAL AND METHODS

Chemicals

Cu_2Zn_2 SOD from bovine erythrocytes, imidazole-2-aldehyde, pyridine-2-aldehyde, TPA, Histopaque 1077 and 1119 and lucigenin were purchased from Sigma, München. TBA, 2-deoxyribose, DTPA and BSA were from Serva, Heidelberg, Xanthine oxidase from cow milk from Boehringer, Mannheim. The Walker 256 carcinosarcoma was kindly donated by Dr. Löhrke, Deutsches Krebsforschungszentrum, Heidelberg. Dr. Peter, Ciba-Geigy, Basel, supplied us with a probe of desferrithiocin, $CuPu(Py)_2$ and $CuPu(Im)_2$ were synthesized according to.[15,16] $Cu(Sal)_2$, $Cu(Ser)_2$ and Cu-thiocin were prepared following the procedures described in refs.[23-25]

Animals

Male Sprague Dawley rats, weighing 250 g, were kept on a standard laboratory diet ad libitum. 1.4×10^4 WS 256 cells, previously raised as ascites, were subcutaneously transplanted on the left upper thigh of each of 120 rats. Blood was taken by venipuncture of the tail vein and assayed for copper concentration, Cu_2Zn_2 SOD activity and PMN response upon TPA activation. Copper chelates or controls were administered i.v. on days 3, 4, 6, 8 and 10 into five or ten rats each, i.t. injections on days 3, 4, 6 and 10 into four rats each.

Analytical

Plasma-copper was quantified on a Perkin Elmer 3030 atomic absorption spectrometer.

The generation of hydroxyl radicals in the presence of various low M_r copper complexes was monitored using the 2-deoxyribose fragmentation assay.[20] The reproducibility of the duplicate determinations was $< \pm 5\%$. Alternatively, when human PMNs were used, replacing $Cu^+_{lig.}/H_2O_2$ as a source of oxygen free radicals, the test system contained per ml: 1×10^5 PMNs, 5 mM 2-deoxyribose and 20 μM copper chelate in O_2-saturated HEPES buffered saline 50 mM pH 7.4 plus 5.6 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgSO_4$. After 10 min. of preincubation at 37°C, the PMNs were activated by the addition of 250 nM TPA and incubated for 45 min. at 37°C. The phagocytes were precipitated by the addition of 0.5 ml TCA 2.8% (w/v) and centrifugation (10.000 g, 10 min., 20°C). 0.5 ml TBA 1% (w/v) were added to the supernatant and the mixture heated to 96°C for 10 min. After cooling to 20°C, TBA-reactive substance was quantified at A_{532}. Duplicate determinations were reproducible better than $\pm 9\%$.

The separation of polymorphonuclear leukocytes from human blood was performed according to.[26] The cells were quantified and adjusted to a final concentration of 1×10^5 PMNs/ml. The viability was 95% as determined by trypan blue exclusion.

Cu_2Zn_2 superoxide dismutase activity was measured using the lucigenin amplified xanthine/xanthine oxidase chemiluminescence assay.[18] 1 ml contained: rat plasma 1:1000, 0.1 mM lucigenin, 0.1 mM DTPA, 180 nM xanthine oxidase in O_2-saturated HEPES buffered saline 50 mM pH 7.4. After 10 min. of preincubation at 37°C, 20 μM xanthine were added and the resulting photon emission recorded and integrated in a Berthold LB 9505 C luminometer. The observed inhibition caused by plasma SOD was compared with a calibration curve evaluated under the same assay conditions using Cu_2Zn_2 SOD from bovine erythrocytes. 1 SOD unit is defined as copper concentration required for a 50% inhibited chemiluminescence [I_{50}] under the specified conditions and is equivalent to 5 nM copper. The reproducibility of triplicate determinations was better than $\pm 4\%$.

The oxidative burst dependent phagocytic response was monitored in whole blood in the presence of lucigenin as a chemiluminogenic amplifier. 1 ml contained: 0.1 mM lucigenin, 250 nM TPA and rat blood diluted 1:100 with 50 mM HEPES buffered saline pH 7.4 plus 5.6 mM glucose, 1 mM $MgSO_4$ and 1 mM $CaCl_2$. The chemiluminescence at 37°C was recorded and integrated on a Berthold LB 9505 C luminometer over 8 min. The reproducibility of triplicate determinations reached $\pm 7\%$.

RESULTS AND DISCUSSION

Generation of Hydroxyl Radicals by Low Molecular Weight Superoxide Dismutase Mimics in the Presence of Hydrogen Peroxide and Ascorbate

$Cu^{+}_{aq.}$ can effectively catalyze the generation of ·OH radicals in a Fenton type of reaction at a bimolecular rate constant of $k_2 = 1 \times 10^9\ M^{-1}s^{-1}$ (eq. 1).[27]

$$Cu^{+}_{aq.} + H_2O_2 \rightarrow Cu^{2+}_{aq.} + \cdot OH + OH^- \qquad (1)$$

Alternatively, the mechanism of this reaction might proceed via a peroxy-adduct where copper is transiently oxidized to Cu(III) $3d^8$.[19] This cupryl intermediate can be regarded as a complex of Cu^{2+} with co-ordinated ·OH (eq. 2).[28] Its properties clearly differ from those of freely-diffusing hydroxyl radicals and might therefore account for the site-specific damage of Cu^{+}/H_2O_2 often seen in biological systems.[29]

$$Cu^{+}_{eq.} + H_2O_2 \rightarrow Cu(OH)^{2+}_{aq.} + OH^- \qquad (2)$$

The second order rate constant of eq. (2) is $k_2 = 4.7 \times 10^3\ M^{-1}s^{-1}$.[19] In a cell with an average volume of 10^{-11} liter, the contact of 15 nM Cu^+ (0.1% of intracellular copper[27]) with 100 nM H_2O_2 (intracellular steady-state concentration[27]) would theoretically form 42 or 9.03×10^6 ·OH radicals per second per ml, depending on mechanism (2) or (1). Under the same conditions, only one ·OH radical/cell/sec is generated in the presence of Fe^{2+}/H_2O_2.[27] Usually intracellular copper and iron are coordinated in such a way that redox cycling is avoided and no Fenton reaction can take place. The intracellular presence of stable low M_r copper chelates with SOD mimetic activity, including $CuPu(Py)_2$ or $CuPu(Im)_2$, could significantly increase the formation of ·OH radicals. Their SOD-mimetic reactivity would continuously replenish H_2O_2, the substrate for the Fenton reaction. In rapidly metabolizing cells the steady-state concentrations of superoxide and hydrogen peroxide are enhanced. A cyclic SOD- and Fenton-like generation of activated oxygen species is expected to have a deleterious effect on any tumor cell. At the same time, the extracellular stimulation of phagocytic activities of polymorphonuclear leukocytes yield in ad-

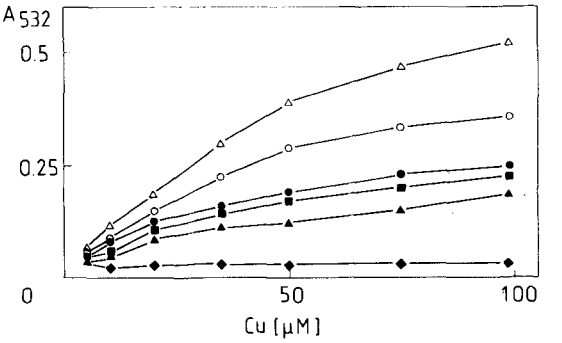

FIGURE 2 Fragmentation of 2-deoxyribose in the presence of various concentrations of differently coordinated low molecular weight copper chelates with superoxide dismutase mimetic activity. △ $CuPu(Im)_2$; ○ $Cu(Sal)_2$; ● $CuPu(Py)_2$; ■ $CuSO_4$ and $Cu(Ser)_2$, resp.; ▲ Cu-thiocin; ◆ Cu_2Zn_2 superoxide dismutase or apo-chelates ($Pu(Im)_2$, $Pu(Py)_2$, salicylate, serinate). For assay conditions see Material and Methods.

dition to superoxide up to 25 μM H_2O_2.[30] Either phagocytic activity and the pronounced Cu(I)/Cu(II) redox chemistry of the stable Cu-coordination compounds would cooperatively increase the concentration of transiently formed reactive species.

The ·OH radical generating properties of our dischiffbases $CuPu(Py)_2$ and $CuPu(Im)_2$ were monitored using the deoxyribose fragmentation assay[20] and compared to that of $CuSO_4$, Cu_2Zn_2 superoxide dismutase, $Cu(Sal)_2$ and $Cu(Ser)_2$ (Figure 2).

The ·OH producing reactivity was most pronounced in the presence of 100 μM $CuPu(Im)_2$, exceeding that of $CuSO_4$ by 130%. $Cu(Sal)_2$ increased the TBA reactive substance by 43% and $CuPu(Py)_2$ by 14%, whereas an inhibition of 22% was noticed with Cu-Thiocin, a converted siderophore originating from Streptomyces antiobioticus.[25] $Cu(serinate)_2$ behaved like $CuSO_4$. As expected, the apochelates ($Pu(Im)_2$, $Pu(Py)_2$, salicylate, serinate and thiocin) or Cu_2Zn_2 superoxide dismutase reacted like the control where copper was omitted.

Generation of Hydroxyl Radicals by Various SOD Mimics in the Presence of Activated Polymorphonuclear Leukocytes

PMNs release significant amounts of copper-thioneins upon activation.[31] Due to their high content of cysteines (> 30 mol%) these ubiquitous low M_r proteins can scavenge hydroxyl radicals very effectively ($k_2 = 1 \times 10^{12}\,M^{-1}s^{-1}$).[32] The release of thioneins during activation protects polymorphonuclears against their own oxidative damage.[38]

Thus, compared to inorganic assay systems, the ·OH dependent deoxyribose fragmentation is considerably diminished, but increases to 74% in the presence of $CuSO_4$ or $Cu(Ser)_2$ (Table I). When $CuPu(Im)_2$ substituted $CuSO_4$ as Fenton catalyst, a 146% increase in TBA reactive substance was detectable. $CuPu(Py)_2$ and $Cu(Sal)_2$ (126% and 118% increase, resp.) behaved similar. The apo-chelates or superoxide dismutase inhibited the degradation insignificantly by 4%.

Anticarcinogenic Reactivity of Dischiffbase Coordinated SOD Mimics in Sprague Dawley Rats Suffering from a Walker 256 Carcinosarcoma

Total remissions and mortality The Walker 256 carcinosarcoma is a fast growing experimental tumor model. After an initial lag phase of diminished growth, the average tumor size doubles every two days and reaches approximately 15% of the

TABLE I

Hydroxyl radical dependent degradation of 2-deoxyribose in the presence of 1×10^5 polymorphonuclear leukocytes and various copper chelates (20 μM each). For detailed experimental conditions see Material and Methods

treatment	TBA-reactive susbtance A_{532}	% change compared to control
Control	0.192	
not activated	0.018	
$CuSO_4$	0.334	+74
$CuPu(Im)_2$	0.472	+146
$CuPu(Py)_2$	0.434	+126
$Cu(Sal)_2$	0.419	+118
$Cu(Ser)_2$	0.336	+75
Cu-thiocin	0.294	+53

total body weight until the animals die on days 13 ± 2. When four doses of 5 μmol/kg $CuPu(Im)_2$ and $CuPu(Py)_2$, resp., were injected intratumorally on day 3, 4, 6 and 10, 75% of the rats recovered from this sarcoma (Table II).

Similar results were obtained with 0.5 μmol/kg i.t. aplicated Cu-dischiffbases and even 50 nmol/kg $CuPu(Im)_2$ were still sufficient to cure 50% of the animals, whereas this substantially low concentration did not lead to any total remissions in the case of $CuPu(Py)_2$. The tumor growth in non-recovering animals was considerably dimi-

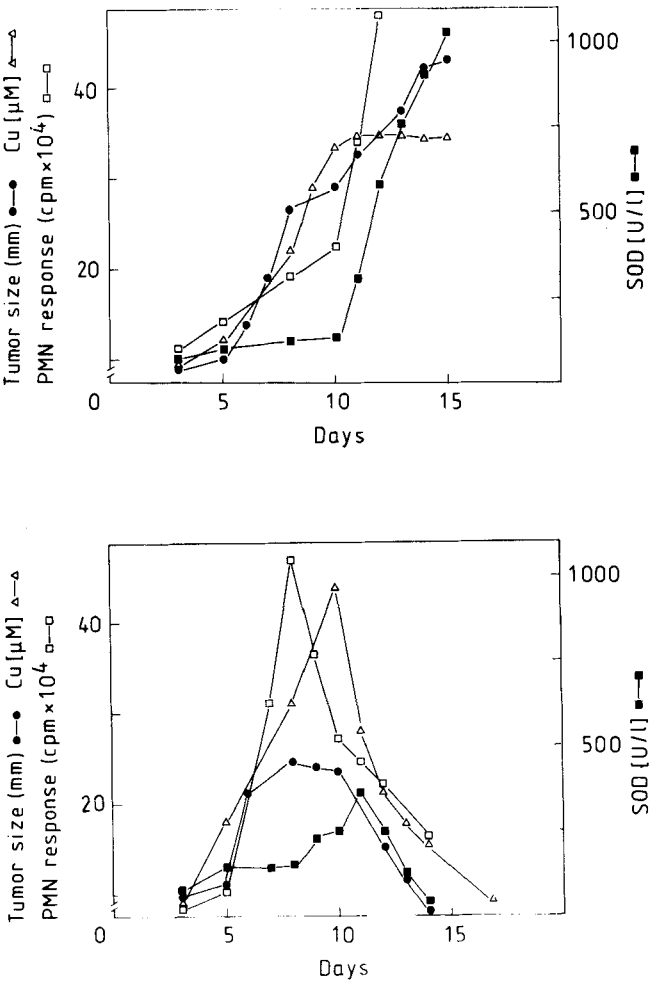

FIGURE 3a–e Time-dependent changes of average tumor size ●, copper concentration in plasma △, Cu_2Zn_2 superoxide dismutase activity in plasma ■ and TPA-response of polymorphonuclear leukocytes and macrophages in unseparated blood □ of Sprague Dawley rats suffering from a Walker 256 carcinosarcoma during the treatment with Cu-dischiffbases, $CuSO_4$, $Pu(Im)_2$ saline. 3a. Saline treated control; 3b. 6.7 μmol/kg $CuPu(Py)_2$; 3c. 6.7 μmol/kg $CuPu(Im)_2$; 3d. 20 μol/kg $Pu(Im)_2$; 3e. 20 μmol/kg $CuSO_4$. For detailed experimental conditions see Material and Methods.

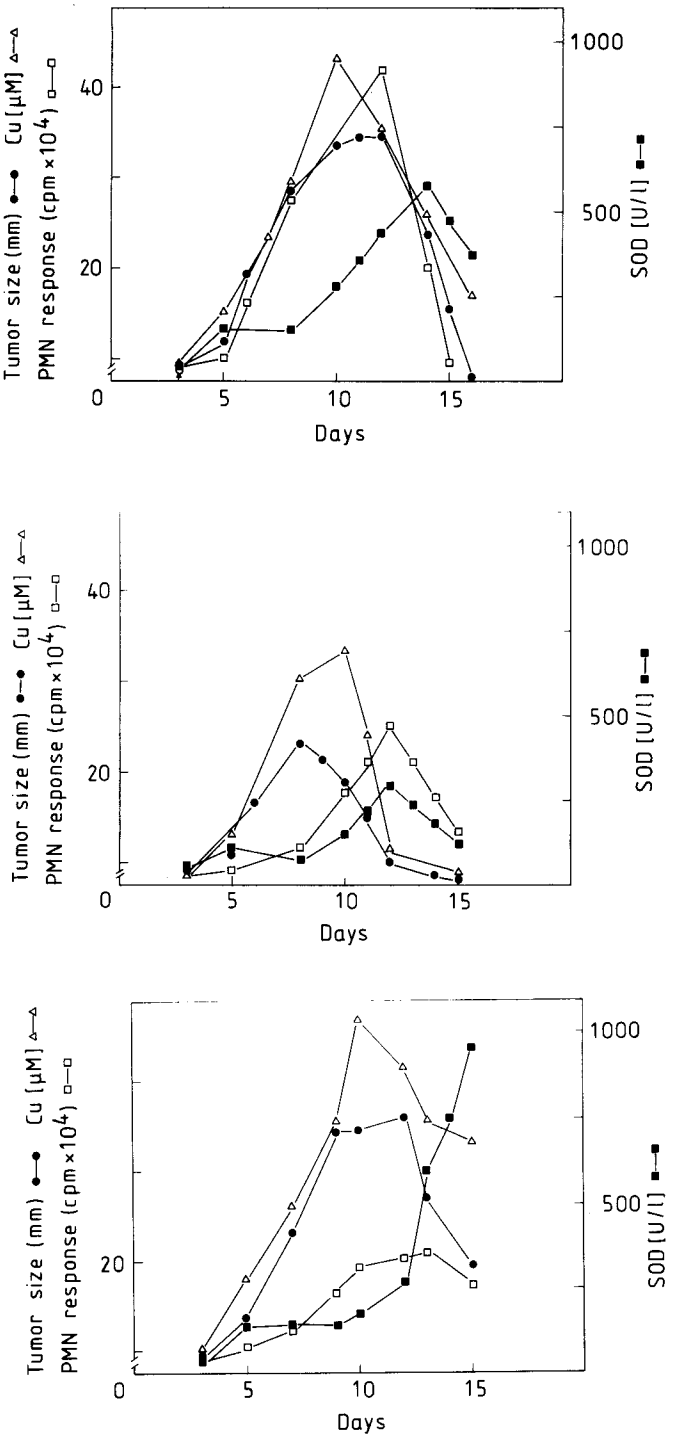

TABLE II
Intratumoral application of Cu-dischiffbases into Sprague Dawley rats implanted with Walker 256 carcinosarcoma cells. Total remissions, mortality and mean survival times

treatment	concentration [μmol/kg]	total remissions %	mean survival time of unrecovered rats (days)	n/m
Saline		13*	13	8/7
$CuPu(Im)_2$	5	75	17	4/1
$CuPu(Im)_2$	0.5	75	16	4/1
$CuPu(Im)_2$	0.05	50	15	4/2
$CuPu(Py)_2$	5	75	16	4/1
$CuPu(Py)_2$	0.5	50	14	4/2
$CuPu(Py)_2$	0.05	0	14	4/4
$CuSO_4$	5	0	11	4/4
$Pu(Im)_2$	5	25	14	4/3
$Pu(Py)_2$	5	0	13	4/4

n number of animals
m mortality
*one spontaneous remission.

nished and the mean survival time prolonged by 1–4 days (Table II). The ligands of $CuPu(Py)_2$, $Pu(Py)_2$, had no significant effect on tumor growth and host survival and behaved similar to saline treated rats. One total necrosis was observed with $Pu(Im)_2$. When $CuSO_4$ was administered intratumorally an interesting phenomenon was observed. The WS 256 proliferation was enhanced by 30%, leading to earlier deaths of the animals as compared to the saline control.

As Cu^{2+} is known to induce the synthesis of metallothionein, the intracellular increase in antioxidative capacities might possibly explain this observation. Enhanced antioxidative defences are often noticed in malignant cells with a concommitant drug resistance.[33–35] Unlike the single cysteine sulphur of glutathione, metallothionein has 20 cysteine residues, which react as a powerful intracellular antioxidant in rapidly-proliferating cells. The intracellular metallothionein concentration can amount quite frequently to 0.2 mM.[32] When the equivalency of reactive thiolate-sulphurs is compared with either glutathione and metallothionein, the effective intracellular thiol concentration of metallothionein is 4 mM to that of 3 mM, when GSH serves as thiol donor. At the same time the bimolecular rate constant of metallothionein with hydroxyl radicals is approx. 100 times faster than that of glutathione ($k_2 = 8.8 \times 10^9\,M^{-1}s^{-1}$).[27]

By way of contrast, the intravenous application of five doses of 20 μmol/kg $CuSO_4$ (Figure 3e) did not enhance the tumor growth observed upon intratumoral administration, suggesting that negligible amounts of Cu^{2+} will reach the tumor that way. Serum albumin binds tenaciously Cu^{2+}, which is rapidly eliminated by the liver ($t_{1/2}$ = 10 min.).[36] Significantly higher intratumoral copper concentrations can be expected when the stable chelates $CuPu(Py)_2$ and $CuPu(Im)_2$ are used. Thus, 60% of total remissions were observed, when 5 doses of 6.7 μmol/kg $CuPu(Py)_2$ were administered i.v. to 10 rats each and 20% in the case of $CuPu(Im)_2$ (Table III). At the same time $CuPu(Im)_2$ diminished the proliferation of the sarcoma and enhanced the mean survival time by 3 days. Administration of larger amounts of the compounds had no additional effect.

TABLE III
Intravenous administration of Cu-SOD mimics into Sprague Dawley rats implanted with Walker 256 carcinosarcoma cells. Total remissions, mortality and mean survival times

treatment	concentration [μmol/kg]	total remissions %	mean survival time of unrecovered rats (days)	*n/m*
Saline		0	13	*10/10
$CuPu(Py)_2$	6.7	60	16	*10/ 4
$CuPu(Im)_2$	6.7	20	16	*10/ 8
$CuSO_4$	20.0	10	15	*10/ 9
$Pu(Py)_2$	20.0	0	14	5/ 5
$Pu(Im)_2$	20.0	60	15	*10/ 4

n number of animals
m mortality
*two separate experiments

Our results confirm those obtained with human K 562 erythroleukemia cells, where $CuPu(Py)_2$ and $CuPu(Im)_2$ caused significant depletions of intracellular thiolate-dependent antioxidative defences.[6,37] Interestingly, 60% of the rats recovered from the sarcoma, previously treated with five doses of 20 μmol/kg $Pu(Im)_2$, the parent compound of $CuPu(Im)_2$ (Figure 3d). As $Pu(Im)_2$ chelates copper 10 times better than albumin,[16] the corresponding SOD-mimic $CuPu(Im)_2$ can be formed *in situ* in the presence of copper. It should be emphasised that the copper level rose by 325% in the plasma of saline treated tumor controls.

Moreover, $Pu(Im)_2$ reduced this elevated copper concentration to the level of healthy non-tumor bearing rats (Table IV). Thus, a diminished copper availability to the rapidly-proliferating tumor tissue most likely contributes to its antitumor reactivity.

Plasma Copper Concentration, Superoxide Dismutase Activity and PMN Responses

It is well known that during inflammatory events and carcinogeneses the copper plasma concentration is elevated by 300% or more.[36] Accordingly, the linear increase starting at 8 ± 1 μM Cu was completed within days 6 to 10 after the WS 256 transplantation. Saturation remained at 34 ± 5 μM Cu until death (Figure 3e). Upon intravenous application of five doses of 6.7 μmol/kg $CuPu(Py)_2$ and $CuPu(Im)_2$, resp., this concentration increased to 44 ± 3 μM on days 10–11 and was progressively diminished within 5 days after the last injection to the level of healthy non-tumor bearing rats (Figures 3b, c; Table IV). The normalizing copper plasma concentration paralleled the total remissions observed. By way of contrast, the plasma copper content of $CuSO_4$ treated rats did not normalize but stayed at the level of the control until death (Figure 3e). The plasma Cu_2Zn_2 superoxide dismutase activity was measured at different times during the time-course of carcinogenesis. Similar to the copper content, the plasma SOD activity correlated well with increasing or declining tumor sizes. Unlike with healthy animals, the extracellular SOD activity was elevated more than 30 times, reaching 1080 ± 130 U/l on day 15 in the saline treated tumor control group (Figure 3a). In comparing the SOD and copper concentrations, approximately 15% of the plasma-Cu can be attributed to superoxide dismutase. It should be emphasized that this activity was totally leveled off, when 1 mM CN^- was added to

the test system, known to effectively bind to the active centre of Cu_2Zn_2 superoxide dismutase.[39]

The administration of $CuPu(Py)_2$, $CuPu(Im)_2$ and $Pu(Im)_2$, resp., increased the plasma Cu_2Zn_2 superoxide dismutase concentrations as well (Figures 3b–d), but, unlike the saline- or $CuSO_4$ control (Figures 3a, e), where the SOD levels remained elevated until death, this reactivity rapidly declined upon recovery to concentrations measured in healthy animals (32 ± 12 U/l). $Pu(Py)_2$ did not display any tumoricidal reactivity (Table IV). Usually cytosolic Cu_2Zn_2 SOD can not be detected extracellularly.[39] Upon membrane damage this enzyme is released and can be used as sensitive parameter to accurately quantify the vitality of cell cultures *in vitro*. *In vivo* elevated SOD levels are reported in patients suffering from hepatitis.[40,41] In our study hepatotoxicity was excluded, as the i.v. administration of 6.7 μmol/kg $CuPu(Py)_2$ and $CuPu(Im)_2$, resp., into healthy rats did not result in any significant release of SOD into the blood. Additionally sorbitol dehydrogenase activity was determined.[42] 25 ± 10 SDH U/l were measured in the plasma of $CuPu(Py)_2$, $CuPu(Im)_2$ and saline treated rats, respectively. At the same time no significant haemolysis of erythrocytes was observed. Often euoxic peripheries of tumor tissues have been reported to possess elevated Cu_2Zn_2 SOD activities.[2] In comparing the above controls and considering that the WS 256 tumor mass takes some 15% of the total body weight of the animals, the elevated plasma SOD concentrations most likely were attributable to Cu_2Zn_2 SOD originating from necrotic tumor tissue. Unlike human phagocytes rat PMNs do not circulate in the blood. They are predominately located in the spleen until an adequate signal triggers the infiltration into inflamed areas (Figure 3a). Thus, the chemiluminogenic response upon TPA activation increased linearly with time and was elevated more than 11 times in tumor-bearing rats when compared to healthy animals (Table IV). Much to our surprise, the application of $CuPu(Py)_2$ or $CuPu(Im)_2$ lead to a faster activation (3–4 days earlier than the saline control) and more pronounced maximum response of the blood phagocytes. At the same time both groups showed signs of acute inflammation in areas of tumor necrosis by the presence of PMNs and macrophages in exudate taken at biopsy. The phenomenon of enhanced stimulation of phagocytic activities in the presence of dischiffbase coordinated SOD mimics is probably most important in this fast growing tumor model and has been reported for the antitumor agent CuDIPS as well.[3]

This reactivity might be due to an enhanced peroxidation of membrane lipids,

TABLE IV

Tumor size, copper concentration and Cu_2Zn_2 superoxide dismutase activity in plasma as well as PMN response upon TPA activation in unseparated blood on day 14 in WS 256 SD rats

treatment	tumor size (mm)	SOD [U/l]	Cu [μM]	PMN response (cpm × 10^4)	*n*/*m*
Saline	43 ± 5	920 ± 120	34 ± 5	57 ± 11	10/10
No tumor		32 ± 12	8 ± 1	5 ± 1	5/ 0
$CuPu(Py)_2$	* 0	* 50 ± 10	*15 ± 2	*17 ± 6	10/ 4
$CuPu(Im)_2$	*24 ± 3	*560 ± 60	*25 ± 4	*21 ± 6	10/ 8
$Pu(Py)_2$	***45 ± 8	*640 ± 60	***35 ± 4	***61 ± 18	5/ 5
$Pu(Im)_2$	* 8 ± 4	*200 ± 14	* 9 ± 1	*18 ± 3	10/ 4
$CuSO_4$	*23 ± 10	**750 ± 80	***34 ± 6	*20 ± 6	10/ 9

n number of animals; *m* mortality; *$p < 0.001$; **$p < 0.01$; ***not significant

caused by hydroxyl radicals generated in the presence of $CuPu(Py)_2$ and $CuPu(Im)_2$. During lipid peroxidation a considerable amount of 4-hydroxynonenal is produced. This aldehyde is a potent chemo-attractant for polymorphonuclear leukocytes at concentrations around 100 nM or less.[43]

Upon recovery, the elevated phagocytic activity in the presence of Cu-dischiffbases declined rapidly compared to that observed in healthy animals and correlated well with normalizing SOD- and copper concentrations. For comparative reasons, tumor size, plasma SOD-, and Cu-concentrations as well as PMN activity upon TPA activation in whole blood are summarized for day 14 in Table IV. Statistical analysis was performed using Student's *t*-test.

No recidives were observed in surviving animals within 14 days after recovery. Autopsies were performed on day 28. No pathological changes of liver, lung or heart (locations where metastases are occasionally found with WS 256) were detectable. The original tumor site was filled with a yellowish fat-like substance.

CONCLUSIONS

Stable low molecular weight Cu-dischiffbases with superoxide dismutase mimetic activity, including $CuPu(Py)_2$ and $CuPu(Im)_2$, are efficient antitumor agents in the Walker 256 carcinosarcoma and in the human K 562 erythroleukemia cell line.[6] When acetate type antitumor agents, including CuDIPS, were used, up to 18 daily doses of 50 μmol/kg were required to increase the mean survival time of mice implanted with Ehrlich carcinoma cells.[3] Only five daily doses of 5 μmol/kg $CuPu(Py)_2$ sufficed to totally recover 60% of male Sprague Dawley rats from the fast growing Walker 256 carcinosarcoma. This pronounced anticarcincogenic reactivity is assigned to both the SOD reactivity and stability of Cu-dischiffbases to survive competitive biological chelation.[16] The SOD-like activity is virtually identical to that of native Cu_2Zn_2 superoxide dismutase[18] and approximately ten times better than that of CuDIPS. The Cu(I)/Cu(II) redox cycling activity of Cu-dischiffbases continuously replenishes hydrogen peroxide that is used as substrate to generate hydroxyl radicals. Subsequently, this results in the depletion of intracellular thiolate-dependent antioxidative defence mechanisms.[37] Moreover, the application of $CuPu(Py)_2$ and $CuPu(Im)_2$, respectively, causes a pronounced activation of blood phagocytes, where the process of tumor phagocytosis is cooperatively supported by dismuting superoxide via hydrogen peroxide to yield highly toxic ·OH radicals. The intra- and extracellularly increased flux of oxygen free radicals eventually leads to tumor necrosis.

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