Antiproliferative Activity of Derivatives of *trans*-Bis(salicylaldoximato)copper(II) *in vitro*. Some *in vivo* Properties of the Parent Compound

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Z. Naturforsch. **41c,** 951–955 (1986); received March 7/May 15, 1986

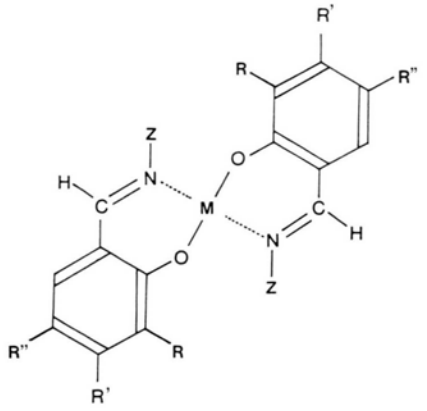
Copper Chelates, Antiproliferative Activity, Pyridoxal Hydrochloride, Toxicity, L1210 Leukemia Cell Culture

Several derivatives and analogs of the recently reported antiproliferative and antitumor agent *trans*-bis(salicylaldoximato)copper(II) ($CuSAO_2$) have been prepared and tested for antiproliferative activity against L1210 leukemia cells *in vitro*. The salicylaldimine analog of $CuSAO_2$ had a very strong antiproliferative activity, the 2-day IC_{50} value being lower than 3 μg/ml. The 2,3-dihydroxybenzaldoxime analog was equally active with $CuSAO_2$, while the corresponding 2,5-dihydroxy derivative had a slightly lower activity. The 2,3,4-trihydroxybenzaldoxime derivative had a much lower activity than had the dihydroxybenzaldoxime derivatives. The zinc(II) analog of $CuSAO_2$ had only a low antiproliferative activity. The ligand of $CuSAO_2$, salicylaldoxime, resembles pyridoxal oxime, a vitamin B_6 antagonist and a powerful inhibitor of pyridoxal kinase. An attempt to reduce the toxicity of $CuSAO_2$ *in vivo* with pyridoxal hydrochloride led to increased toxicity.

Introduction

We have recently shown that *trans*-bis(salicylaldoximato)copper(II) ($CuSAO_2$; Ia, Z = OH, M = Cu, R = R′ = R″ = H) and its derivative *trans*-bis(2,4-dihydroxybenzaldoximato)copper(II) ($CuRES_2$; Ib, Z = OH, M = Cu, R = R″ = H, R′ = OH) have a very strong antiproliferative activity against tumor cells *in vitro*. Both compounds were shown to totally arrest the proliferation of leukemia L1210 and Ehrlich ascites carcinoma cells in the concentration of about 5 μg/ml [1]. The antiproliferative activity is a property of the intact chelates only. The ligand part of $CuSAO_2$, salicylaldoxime, had no antiproliferative activity even in high concentrations, and free copper(II) salts had only a very limited activity [1].

Further, it was shown that $CuSAO_2$ has strong antitumor activity against Ehrlich ascites carcinoma in mice *in vivo* [2]. Because of these promising results, further studies on the compounds and their derivatives are warranted. Screening of new derivatives is especially interesting, not only because it may lead to the discovery of new active compounds, but also because it may reveal possible structure-activity correlations. Such correlations may in turn be valuable in the elucidation of the mechanism of action of the compounds, and may also form the basis for a more rational search of derivatives of higher activity. Therefore, we have now synthesized some derivatives and analogs of $CuSAO_2$ and $CuRES_2$, and tested them against leukemia L1210 cells *in vitro*. We also report here the results of some preliminary *in vivo* experiments on $CuSAO_2$.



I

Materials and Methods

Chemicals

$CuSAO_2$ [3] and the corresponding Zn(II) chelate (**Ie**) [4] were prepared as previously described. Compound **Id** (see Table I for substituents) was prepared according to the method of Pfeiffer *et al.* [5]. Compound **II** was prepared essentially according to the method of Dubský and Sokol [6]. The syntheses of other compounds will be described elsewhere. Analytical data on the compounds tested *in vitro* are given in Table I.

Pyridoxal hydrochloride (biochemical grade) and dimethyl sulfoxide (spectroscopical grade or scintillation grade) were obtained from E. Merck.

In vitro screening

Mouse L1210 leukemia cells were grown at 37 °C in Gibco's medium RPMI 1640 supplemented with

Abbreviations: $CuRES_2$, *trans*-bis(2,4-dihydroxybenzaldoximato)copper(II), also known as *trans*-bis(β-resorcylaldoximato)copper(II); $CuSAO_2$, *trans*-bis(salicylaldoximato)copper(II); EAC, Ehrlich ascites carcinoma.

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5% (v/v) of pooled human serum (Finnish Red Cross Transfusion Service), 2 mM glutamine, and 50 mg of the sodium salt of penicillin G and 50 mg of streptomycin per litre. The cells were counted using a particle counter (Coulter Electronics, model Industrial D). – For each test series, a confluent cell suspension was diluted, the cells were counted, and 5 ml aliquots of the suspension were grown in tightly closed disposable culture tubes of 10 ml volume. The test substances were added as solutions or suspensions in dimethyl sulfoxide (E. Merck) (25 µl/5 ml). Controls and solvent controls were employed. The number of cells in each tube was counted after 1 and 2 days. For each compound and concentration tested, the value of the growth ratio

$$Y = \frac{N - A}{C - A}$$

was calculated, where N is the number of cells in the tube containing the test substance, C is the mean number of cells in the solvent control tubes, and A is the initial value. Percentage inhibition, obtained by subtracting the value of Y from 100%, was plotted against concentration, and approximate concentrations which inhibit growth to 50% of control growth (IC_{50}) were determined graphically.

In vivo experiments

NMRI mice were obtained from the Department of Pharmacy of the University of Helsinki. They were housed at ordinary room temperature with a 12 h light-dark cycle and were given a standard laboratory diet and tap water *ad libitum*.

The detergent solution used in the pyridoxal experiment was prepared by dissolving 100 µl of Tween 20® (polyoxyethylenesorbitan monolaurate; Ph. Eur. grade) in 100 ml of 0.9% aqueous NaCl solution.

Results and Discussion

In vitro screening of derivatives of $CuSAO_2$

Most of the $CuSAO_2$ derivatives and analogs tested had the general formula **I**. Table I shows the results of screening in cell culture. Usually, four different concentrations were tested for each compound.

The salicylaldimine analog (**Ic**) of $CuSAO_2$ was found to have a considerable antiproliferative activity. During the first day, its IC_{50} value was as low as ca. 3–4 µg/ml, the 2-day value being even lower. The compound is a potential candidate for testing *in vivo*. The results given in Table I were obtained with a freshly prepared solution of **Ic**. The compound is unstable in dimethyl sulfoxide, as indicated by color changes, and when the solutions are allowed to stand for several days, the antiproliferative activity is lost almost totally. The corresponding zinc chelate (**Id**) had a much weaker activity.

We have previously shown that the nickel(II) analog of $CuSAO_2$ has only a very low antiproliferative activity, while the activity of the cobalt(II) analog is almost as high as that of $CuSAO_2$ [1]. The

Table I. Results of screening of derivatives and analogs of $CuSAO_2$ against leukemia L1210 cells *in vitro*[a].

Compound	Structure Z	M	R	R′	R″	IC_{50} [µg/ml] 1 day	2 days	Formula	Analytical data Calculated [%] C	H	N	Cu	Found [%] C	H	N	Cu
Ic	H	Cu	H	H	H	3–4	< 3	$Cu(C_7H_6NO)_2$			9.22	20.9			9.09	20.5
Id	H	Zn	H	H	H	10–20	5–10	$Zn(C_7H_6NO)_2$								
Ie[b]	OH	Zn	H	H	H	5–20	> 20	$Zn(C_7H_6NO_2)_2 \cdot H_2O$								
If	OH	Cu	OH	H	H	4	4	$Cu(C_7H_6NO_3)_2$	45.72	3.29			45.05	3.21		
Ig	OH	Cu	H	H	OH	2.5–5	2.5–5	$Cu(C_7H_6NO_3)_2 \cdot H_2O$	43.58	3.66			43.61	3.60		
Ih	OH	Cu	OH	OH	H	18	10	$Cu(C_7H_6NO_4)_2$	42.06	3.03			41.47	3.00		
Ii	$(CH_2)_2OH$	Cu	H	H	H	17–50	9	$Cu(C_9H_{10}NO_2)_2$	55.16	5.14	7.15	16.2	55.86	5.20	6.84	15.2
Ij	$(CH_2)_3OH$	Cu	H	H	H	10–20	< 9	$Cu(C_{10}H_{12}NO_2)_2$				15.1				15.0
Ik	$(CH_2)_3OH$	Cu	OH	H	H	5–10	12	$Cu(C_{10}H_{12}NO_3)_2 \cdot H_2O$	51.11	5.58			50.71	5.42		
II						> 20	> 20	$Cu(C_{16}H_{14}N_2O_2)$	58.26	4.28	8.50	19.3	58.50	4.36	8.16	18.02

[a] The IC_{50} values should be considered as rough estimates only. Determination of accurate values was not deemed important for the purposes of this study. The IC_{50} values of $CuSAO_2$ and $CuRES_2$ are between 3 and 4 µg/ml.

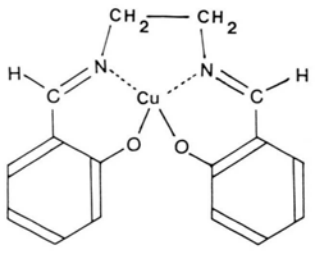
[b] This compound was added to the cell culture as a suspension.

present results indicate that the corresponding zinc(II) chelate (**Ie**) is only a weak inhibitor of tumor cell proliferation. Copper and cobalt compounds are usually more prone to redox reactions than are nickel and zinc compounds. Thus, taken together, the results suggest that redox reactions may have a role in the antiproliferative activity of $CuSAO_2$ and related compounds. Other explanations for the low activity of the nickel and zinc chelates cannot, however, be ruled out. For example, the low activity of the zinc chelate may at least in part be due to its very low solubility.

The 2,3- and 2,5-dihydroxybenzaldoxime derivatives (**If**) and (**Ig**) are isomers of the previously studied $CuRES_2$ [1]. All of these compounds have a very high antiproliferative activity against L1210 leukemia cells. The *in vitro* dose-response curves of $CuRES_2$ [7] and **If** rise very steeply between 3 and 5 μg/ml, just like that of $CuSAO_2$ [7], and reach ca. 90% inhibition at about 5 μg/ml. In the case of **Ig**, however, the rise of the curve is somewhat less steep, and higher concentrations are needed for 90% inhibition. The antiproliferative activity of the 2,3,4-trihydroxybenzaldoxime derivative (**Ih**) is clearly lower than that of the dihydroxybenzaldoxime derivatives studied. Even at the concentration of 20 μg/ml, only 60% inhibition was achieved with this compound.

Hodnett and coworkers have shown that certain cobalt(II) complexes of Schiff bases of salicylaldehyde and aminoalcohols have antitumor activity [8–10]. The chelate of the Schiff base of salicylaldehyde and 3-amino-1-propanol appeared to have especially good antitumor activity [8]. Also some related Schiff bases in their own right slow tumor growth [10–14]. The cobalt complexes reported by Hodnett *et al.* are structurally related to $CuSAO_2$. Therefore, we prepared the copper(II) chelates (**Ii**) and (**Ij**) of the salicylaldehyde Schiff bases of 2-amino-1-ethanol and 3-amino-1-propanol, respectively. The chelate **Ik** of the Schiff base formed by 2,3-dihydroxybenzaldehyde and 3-amino-1-propanol was also synthesized. All of these compounds had moderate antiproliferative activity but were clearly less active than $CuSAO_2$.

Compound **II**, the copper(II) chelate of the tetradentate ligand N,N′-bis(salicylidene)ethylenediamine, was the only one of the present $CuSAO_2$ analogs that did not have any measurable antiproliferative activity. There is at least one major difference between the structure of this compound and all



II

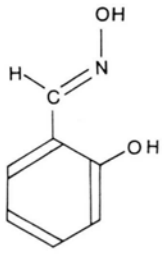
the others tested. Namely, compound **II** cannot exist in the *trans*-configuration that is typical of most $CuSAO_2$ type compounds. This configurational restriction, caused by the structure of the ligand, may well lie behind the total lack of antiproliferative activity. We are currently engaged in the synthesis of other analogs having a similar configurational restriction. Studies on them might reveal, whether *trans*-configuration about the metal ion is essential for the antiproliferative activity of $CuSAO_2$ type compounds. This point is very interesting, because in the case of the well-known and clinically important platinum anticancer drugs, the *cis*-isomers have high antitumor activity while the *trans*-isomers do not show any appreciable activity [15–17]. Hall and Waters [18] have shown that the configuration about the copper atom in **II** is pyramidal in the solid state. It is possible that the compound has this configuration in solution, too. Such non-planarity might also lie behind the lack of biological activity.

In vivo studies on $CuSAO_2$

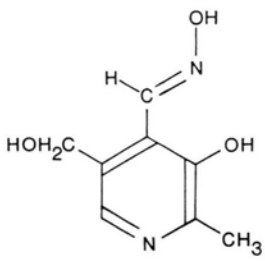
We have previously reported [2] experiments showing that $CuSAO_2$ has a remarkable antitumor activity against EAC *in vivo*. In those experiments, some $CuSAO_2$-treated mice were totally cured, being still alive and apparently healthy 101 days after the injection of EAC cells. Four of those mice were then used for a preliminary immunity test, the results of which are reported here. Each of the test mice (weighing 28–35 g) and each of the ten control mice (weighing 24–34 g, mean 29.7 ± 2.58 g) were given i.p. 0.18×10^6 EAC cells. The mean survival time of the controls was 22.1 ± 2.2 days. Two of the cured $CuSAO_2$-treated mice died of EAC (survival times 20 and 21 days). The other two were resistant and did not take EAC. No increase in their body weights was

detected during 40 days. These very preliminary results are in line with those of Kimoto and coworkers [19], who treated EAC-inoculated mice with a combination of sodium ascorbate and the copper(II) chelate of glycylglycylhistidine and found that those mice that survived over 60 days, were resistant to a new EAC inoculum. Therefore, further studies on the immunological consequences of treatment of tumors with $CuSAO_2$ and other Cu(II) compounds are strongly warranted and might give valuable information on the mechanisms of tumor immunity.

The ligand of $CuSAO_2$, salicylaldoxime (**III**), resembles pyridoxal oxime (**IV**), a well-known vitamin B_6 analog and antagonist and an extremely powerful inhibitor of pyridoxal kinase [20–23]. On the other hand, vitamin B_6 deficiency and B_6 antagonists are known to have growth inhibiting and antitumor activity [24, 25]. These data, together with the finding that the side-effects of $CuSAO_2$ (acrodynia and weight loss) [2] resemble those of vitamin B_6 deficiency and of B_6 antagonists [24, 26], led us to explore the possible interaction of $CuSAO_2$ and the B_6 vitamer pyridoxal. It was hoped that pyridoxal could be used as an antidote of $CuSAO_2$.



III



IV

Thus, a preliminary study was performed, in which female NMRI mice were given i.p. either first saline and 30 minutes thereafter $CuSAO_2$ (30 mg kg^{-1}), or first pyridoxal hydrochloride (100 mg kg^{-1}) and then $CuSAO_2$ (30 mg kg^{-1}), or first pyridoxal hydrochloride (100 mg kg^{-1}) and then saline. The treatments were repeated after 7 h. Appropriate vehicle controls were also employed. (For details, see the legend of Fig. 1.) As is evident from Fig. 1, the mice which twice received pyridoxal hydrochloride and $CuSAO_2$, suffered a weight loss approximately equal to that of the animals which twice received saline and $CuSAO_2$, but recovered weight more slowly than the latter. Thus, in the dosage used, pyridoxal hydro-

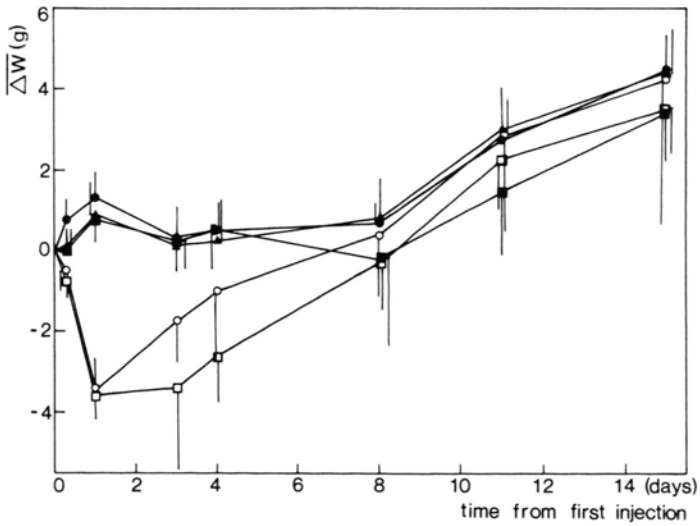


Fig. 1. Mean changes of body weight ($\overline{\Delta W}$) of mice of different groups in the pyridoxal hydrochloride-$CuSAO_2$ test, as compared to the corresponding weights just before the first injection. Female NMRI mice weighing 23.7± 4.4 g were used, divided into five groups of six animals. At the beginning of the experiment, groups I–III were given i.p. 0.9% NaCl solution (0.1 ml/10 g body weight), and 30 min thereafter a second i.p. injection of the same volume was given, group I (●) receiving again 0.9% NaCl solution, group II (▲) receiving the detergent solution (see Materials and Methods) and group III (○) receiving a suspension of $CuSAO_2$ in the detergent solution (30 mg/10 ml, thus 30 mg per kg body weight). Groups IV (□) and V (■) received at the beginning of the experiment i.p. 0.9% NaCl solution containing 1% (w/v) pyridoxal hydrochloride (injection volume 0.1 ml/10 g body weight, thus 100 mg pyridoxal hydrochloride per kg), and 30 min thereafter group IV received $CuSAO_2$ (30 mg/kg body weight) in the detergent solution (0.1 ml/10 g) and group V received 0.9% NaCl solution (0.1 ml/10 g). The first injections were repeated 7 hours after the beginning of the experiment, and the second injections 30 min later. Thereafter no treatments were done. – The bars indicate standard deviations.

chloride did not reduce but, if anything, increased the toxicity of $CuSAO_2$. The administration of saline and pyridoxal hydrochloride did not cause any weight loss. Two of the six mice treated with pyridoxal hydrochloride and $CuSAO_2$ died (during days 3 and 4), while all mice in other groups survived to the end of the experiment (21 days).

In spite of the finding that pyridoxal did not reduce the toxicity of $CuSAO_2$, the possibility cannot be excluded that the mechanism of action of $CuSAO_2$ is based on vitamin B_6 antagonism. B_6 antagonists are namely known, whose effects cannot be reversed by B_6 vitamers [27]. Thus, further studies on the possible role of pyridoxal kinase inhibition in the mechanism of action of $CuSAO_2$ type compounds are warranted. An experiment of the type described above but employing various different timing and dosage schedules might also be warranted.

Acknowledgements

Miss Raija Laine, Mr. Jarmo Pajunen, Miss Gun-Susanne Roth, and Mr. Ehsan Etemadzadeh, M. Sc., are acknowledged for skilful technical assistance. Professor Liisa Ahtee and Associate Professor Juhani Jänne are acknowledged for their cooperation.

H. E. has been financially supported by a grant from the Kymenlaakson rahasto, a regional fund of the Finnish Cultural Foundation.

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