Synthesis, characterization and antitumour studies on *N*-salicyl-*N'*-thiobenzohydrazide and its copper(II) complex

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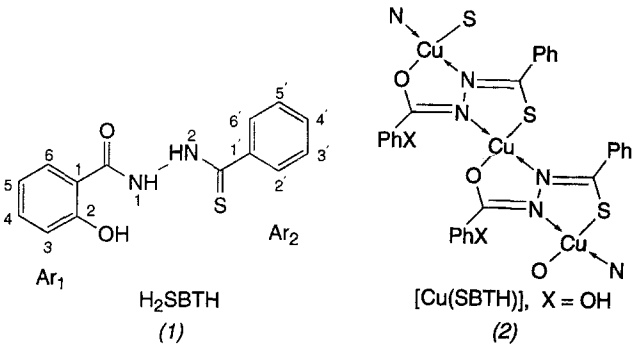
Summary

The new potential tetradentate ligand *N*-salicyl-*N'*-thiobenzohydrazide (H_2SBTH) and its Cu complex, [Cu(SBTH)], have been prepared and characterized by physico-chemical studies. *In vivo* antitumour activity of [Cu(SBTH)] has been tested against breast tumour in C_3H/J strain mice; LD_{50} values were also calculated. The cytotoxicity and antitumour effect of [Cu(SBTH)] is a maximum at 100 mg kg^{-1} body weight injected intraperitoneally in mice carrying breast tumour. *In vitro* results of the ligand and the complex on P-815 (murine mastocytoma) and K-562 (human erythroleukemia cells) indicate that these compounds show significant inhibition on ^{3}H-thymidine and ^{3}H-uridine incorporation in DNA and RNA in these tumour cells. Light microscopic study of the treated tumour mass demonstrated that certain cellular degradation, such as disappearance of mitotic figures, loss in cellular compactness, distortion of nucleus and disruption of cytoplasmic boundaries, take place in complex-treated mice with tumours.

Introduction

For years in anticancer drug design, the focus of scientists has been on organic and inorganic compounds and natural products. During the past decade, after the success with *cis*-dichlorodiammineplatinum(II) (*cis*-platin), a number of derivatives of thiosemicarbazone, such as 3-ethoxy-2-oxobutyraldehyde*bis*(thiosemicarbazone)-copper(II) complexes (Cu-KTS), have been found to exhibit antitumour activity[1,2] by binding with DNA[3]. In addition, the copper complexes of thiosemicarbazide inactivate lambda phage infectivity and transfection by lambda DNA[4], and inhibit the activity of RNA-dependent DNA polymerase and Rous sarcoma virus[5]. Thiosemicarbazones of 1-formylisoquinoline and 2-formylpyridine and their derivatives were also demonstrated to be effective against animal tumours[6,7] at the molecular level by inhibiting the enzyme ribonucleoside diphosphate reductase and synthesis of DNA[8,9]. Although thiohydrazides are structurally quite similar to thiosemicarbazides, scarcity of work on the antineoplastic activity of transition metal complexes of thiohydrazides has prompted us to study the antitumour activity of copper(II) complexes of *N*-salicyl-*N'*-thiobenzohydrazide (H_2SBTH) *(1)*. The present paper reports our investigation on the antitumour activity of the copper(II) complex of (H_2SBTH) *(2)* against breast tumour in mice and *in vitro* testing against P-815 and K-562.



Experimental

Preparation of H_2SBTH (1)

All chemicals used were of analytical reagent or equivalent grade. Salicylic acid hydrazide[10] and carboxymethyl dithiobenzoate[11] were prepared by literature methods. Equimolar amounts of salicylic acid hydrazide and carboxymethyldithiobenzoate, each dissolved separately in 2 and 1 equivalents of aqueous NaOH (2 N), respectively, were mixed and the mixture was left for 2 h at room temperature. The desired product was precipitated by adding dilute AcOH dropwise to this reaction mixture. The product thus obtained was then filtered off, washed with H_2O, dried and recrystallized from hot EtOH. M.p., 200–202 °C. (Found: C, 61.6; H, 4.1; S, 11.7; N, 10.1; $C_{14}H_{12}O_2N_2S$ calcd.: C, 61.8; H, 4.4; S, 11.8; N, 10.3%.)

Preparation of Cu(SBTH) (2)

The complex was prepared by digesting a solution of $Cu(OAc)_2 \cdot H_2O$ (2.00 g) in 50% MeOH (60 cm^3) and the ligand (2.72 g) in MeOH (120 cm^3) and NaOAc (2 g) for *ca.* 15 min. On cooling, the dark brown complex thus obtained was filtered off, washed successively with a H_2O–MeOH mixture and hot EtOH, and dried *in vacuo*. The complex was analysed for its metal, N and S contents following the standard procedure as reported earlier[12]. C, H, N were analysed on a Perkin-Elmer CHN analyser, Model 240C. M.p., 290–292 °C. (Found: Cu, 20.2; C, 49.5; S, 10.1; H, 2.8; N, 8.9; $Cu(C_{14}H_{10}N_2O_2S)$ calcd.: Cu, 19.6; C, 50.4; S, 9.6; H, 3.0; N, 9.6%.)

Physical measurements

The magnetic susceptibility, and i.r. and electronic spectra of the complex were recorded as described earlier[13]. The

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X-band e.s.r. spectrum was obtained in DMSO on a EPR-E-112 spectrometer using DPPH as a $\langle g \rangle$ marker. ^{1}H- and ^{13}C-n.m.r. spectra of the ligand were obtained on a Jeol 90Q multinuclear spectrometer in DMSO-d_6.

In vitro *studies*

The P-815/K-562 cell suspension was prepared in a complete medium (RPMI 1640 medium supplemented with antibiotics, penicillin, streptomycin and 10% heat-inactivated fetal calf serum) at a concentration of 10^6 cells cm^{-3} following a literature method[14]; 2×10^5 cells $well^{-1}$ were added to duplicate wells of a 96-well culture plate (NUNC, Denmark). The cells were treated with test compounds at various doses (1 and 5 μg cm^{-3}) and incubated for 24 h at 37 °C in a CO_2 incubator. In control sets no treatment was given. After 24 h of incubation, the cells were washed thrice with RPMI 1640 culture medium (without serum) by centrifugation (400 *g* for 10 min). The cell pellets were resuspended in 0.2 cm^3 complete medium containing 1 μCi cm^{-3} ^{3}H-thymidine or ^{3}H-uridine and pulse-labelled for 4 h for thymidine and 2 h for uridine. The cells were then washed thrice with phosphate-buffered saline (PBS). The cells were lysed with 1% sodium dodecyl sulfate (SDS) and the lysate was counted for radioactivity in LKB β/-liquid scintillation counter. The percentage inhibition of incorporation was calculated as follows:

$$\% \text{ inhibition} = 1 - \frac{\text{CPM in treated tumour cells}}{\text{CPM in untreated tumour cells}} \times 100$$

In vivo *studies*

C_3H/J female mice, 8–10 weeks old (av. wt 20–22 g), were used in this study. Breast tumour-bearing mice were supplied by the Tata Memorial Cancer Institute, Bombay. The breast tumour cells were transplanted in C_3H/J mice by intraperitoneal (ip) injection of 2×10^6 tumour cells. Two types of tests were carried out: (1), preventive (where the tumour was already well developed in the body); (2) curative (after the transplantation of tumour cells in the normal mice); and the growth of the tumour measured. Six animals were used for each test. The compound suspension was freshly prepared in groundnut oil and was injected only once ip at a dose of 10 mg kg^{-1} body weight. The same volume of sterile groundnut oil (without test compound) was injected in control mice.

Therapeutic effectiveness of the compound against tumour-bearing mice was assessed from their *T/C* percentage which was calculated as follows:

$$\% \; T/C = \frac{\text{Mean life span of treated mice*}}{\text{Mean life span of untreated mice}} \times 100$$

*excluding tumour-free survivors.

Mammary gland tumour system for evalution of antitumour effect

Brown female mice (C_3H/J strain), weighing 20–25 g, obtained from Tata Memorial Cancer Institute, Bombay, were used in this study. Mice maintained in an animal house were given food and water freely. The tumour was transplanted subcutaneously from the white tumour mass from tumour-bearing mice.

Test for toxicity

Animals were transplanted the cells of a mammary gland tumour subcutaneously near the nipples by making air pockets. On day three of transplantation of the mammary gland tumour, the test compound in different doses were injected ip as a single injection, five animals were used per dose level. Toxic doses were estimated on the basis of survivals on the fifth day of injection. The central nervous system (CNS) studies suggest that the drug is remarkably safe having no adverse effect on any of the other parameters studied. However, there was only a slight reduction in aggressive behaviour and response to stimuli in the higher dose range (100 mg kg^{-1}) after 1–2 h. The drug shows marked CNS behavioural effects and a slight CNS depressant effect. The compound has an analgesic effect but no sedative effect. At a higher dose (100 mg kg^{-1}) reduction of aggression only was observed, without any other parameter, being affected.

Histological study

Animals from both control and treated batches were killed at two day intervals up to six days. Tumour tissue with liver, kidney, lung, heart and spleen was fixed in Bouin's fluid (aqueous), dehydrated, kept in cedar wood oil for three days and embedded in paraffin wax, sections of 5 μm were cut, stained in Ehrlich's hemotoxylin eosin stain, dehydrated, cleared in xylene and mounted in DPX solution. Slides were studied under a light microscope. For the tumour cells, Harries's hemotoxylin eosin stain was used and other processes were the same as described above.

Results and discussion

The reaction of H_2SBTH with copper(II) acetate gives a deprotonated complex, Cu(SBTH), which has a high m.p. (290–292 °C) and is generally insoluble in water, ethanol and methanol, but slightly soluble in coordinating solvents like DMSO.

Magnetic moment and electronic spectra

The μ_{eff} value of 1.83 B.M. corresponds to the presence of one unpaired electron. The electronic spectrum of Cu(SBTH) shows a broad band at 17670 cm^{-1} assigned to the envelope of $^2B_{1g} \rightarrow {}^2A_{1g}$, $^2B_{2g}$, ^2E_g transitions, suggesting a square-planar geometry around copper(II)[16].

E.s.r. spectra

The e.s.r. spectrum of the sample at liquid nitrogen temperature shows a high field transition at 3200 G with parallel, perpendicular and average *g* values of 2.172, 2.040 and 2.128, respectively. The trend $g_\parallel > g_\perp > g_e$ (free ion value, 2.0023) shows that the unpaired electron is present in the $d_{x^2-y^2}$ orbital of copper(II)[17].

I.r. spectra

The i.r. spectrum of H_2SBTH in KBr shows a broad band at 3400 cm^{-1}, assigned to the OH group, which remains practically unchanged in Cu(SBTH), indicating the non-involvement of the phenolic oxygen in bonding. The bands due to ν(NH), ν(C=O) and ν(C=S), at 3344, 1630

and 840 cm^{-1}, of the ligand are absent in the spectra of the complex; two new bands appearing at *ca.* 1540 and 695 cm^{-1} are assigned to ν(NCO) and ν(C—S) modes, respectively, suggesting the removal of both the —NH— protons *via* enolization and thioenolization, and bonding of the resulting thiolato sulfur and enolic oxygen with copper(II)[18]. The thioamide bands at 1500, 1300 and 1020 cm^{-1} in the ligand spectrum, due to [β(NH) + ν(CN)], [ν(CN) + β(NH)] and ν(N—N), suffer a positive shift of 66, 30 and 140 cm^{-1}, respectively, in the spectrum of the complex[19,20]. The magnitude of the positive shift in these modes supports the bonding sites indicated above and also suggests the involvement of both hydrazinic nitrogens in coordination. Thus, H_2SBTH behaves as a tetradentate ligand, the bonding sites being the thiol sulfur, enolic oxygen and both the hydrazinic nitrogens.

N.m.r. spectra

The n.m.r. spectrum of free H_2SBTH exhibits resonances for the OH, NH and aromatic protons. The aromatic protons appear as two multiplets between 7.0 and 7.13 and 7.53–8.13 p.p.m. Free H_2SBTH shows resonances at 10.73 and 11.80 p.p.m. for two NH protons adjacent to CS and CO groups, respectively. One signal also appears at 3.40 p.p.m. for phenolic OH. The NH and OH signals disappear on D_2O exchange, thus supporting the above assignments[21].

The ^{13}C-n.m.r. spectrum of the ligand shows 14 signals corresponding to the 14 carbon atoms present in the molecule *(1)*. The assignments for these signals have been made by taking into account the chemical shift values of thiobenzhydrazide (C=S, 192.48; C(1), 138.68; C(2), C(6), 131.37, 129.26; C(3), C(5), 128.88, 128.55; C(4), 126.93) and salicyloyl hydrazone[22]. The signals at δ190.9, 164.3 and 158.5 p.p.m. in H_2SBTH are due to C=S, C=O and C—OH carbons, respectively. The chemical shifts for other carbons are as follows: C(1), 121.19; C(2), 158.52; C(3), 116.69; C(4), 132.57; C(5), 118.05; C(6), 128.67; C′(1), 139.12; C′(2), C′(6), 135.60, 135.11; C′(3), C′(5), 131.21, 130.40; C′(4), 129.429. In view of the paramagnetic nature and insufficient solubility of Cu(SBTH) in DMSO-d_6 it was not possible to record its ^{1}H- and ^{13}C-n.m.r. spectra.

Antitumour screening

The compound shows pronounced antitumour activity against breast tumour at a dose of 10 mg kg^{-1} body weight of mice, with a *T/C* value of 216 for preventive and 193 for curative, which is >125 indicating that the complex is worthy of testing in other tumour systems[15]. More than 25% of the mice survived beyond four months. Furthermore, it was observed that the test compound did not show toxicity even at a dose of 100 mg kg^{-1} body weight. Toxicity at different doses of the test compound was checked and no significant effect or change in various parameters was observed.

The tumour regressed in size upon ip injection of the compound at a dose of 10 mg kg^{-1}. Furthermore, a light microscopic study reveals that the untreated tumour mass shows compact cellular organization with cells having spherical to oval-shaped large nuclei. A large number of mitotic figures can be seen in the untreated mass of tumour. Whereas, following the injection of the test compound, the cellular compactness is lost. The cytoplasm appears to be distorted, the nucleus more transluscent (with chromatin dispersed in the form of granules) and the cytoplasmic boundaries are disrupted. One can observe the infiltration of a large number of leukocytes, lymphocytes and macrophages in the regressing tumour mass after treatment. The antitumour potential of Cu(SBTH) can be compared with the antitumour activity of *cis*-platin, which has also been shown to be effective against some transplantable tumours such as sarcoma 180 in C_3H/J strain mice, which regressed completely after a single ip injection of 10 mg kg^{-1} body weight without any apparent irreversible damage to the host; also upon *cis*-platin treatment, mitolic activity was immediately inhibited and there was increased infilteration of lymphocytes and macrophages in the regressing tumour mass[23,24]. Earlier reports from our department suggest that H_2(SFTH) and Cu(SFTH) complexes show strong antitumour potential[25]. We also tested the compounds for their inhibitory effect on ^{3}H-thymidine and ^{3}H-uridine incorporation in K-562, a human tumour cell line, and P-815, a murine tumour cell line, *in vitro* at 1 and 5 $\mu g\,cm^{-3}$ doses, respectively (Tables 1–4). There was significant inhibition of ^{3}H-thymidine or ^{3}H-uridine incorporation in both K-562 and as P-815 tumour cells; the effect being more pronounced in murine tumour cells. Both *in vitro* and *in vivo* results obtained from the present study show that this is a strong antitumour compound. Furthermore, the results of *in vivo* antitumour activity of Cu(SBTH) show that the present compound has higher activity than thiosemicarbazone and its copper(II) complexes[26,27]. The mechanism of antitumour action of this

Table 1. Percentage inhibition of ^{3}H-thymidine in tumour cell P-815 (murine mastocytoma)

Compound	Dose and inhibition 1 $\mu g\,cm^{-3}$	5 $\mu g\,cm^{-3}$	Inhibition (%) 1 $\mu g\,cm^{-3}$	5 $\mu g\,cm^{-3}$
H_2SBTH	6932	9985	53.67	33.26
Cu(SBTH)	7289	12590	51.28	15.85
Control	14693	–	–	–

Table 2. Percentage inhibition of ^{3}H-uridine incorporation in tumour cells P-815 (murine mastocytoma)

Compound	Dose and inhibition 1 $\mu g\,cm^{-3}$	5 $\mu g\,cm^{-3}$	Inhibition (%) 1 $\mu g\,cm^{-3}$	5 $\mu g\,cm^{-3}$
H_2SBTH	13089	15832	75.68	70.59
Cu(SBTH)	10132	12282	81.18	77.18
Control	53842	–	–	–

Table 3. Percentage inhibition of ^{3}H-thymidine in tumour cells K-562 (human erythroleukemia)

Compound	Dose and inhibition 1 $\mu g\,cm^{-3}$	Inhibition (%) 1 $\mu g\,cm^{-3}$
H_2SBTH	17316	40.25
Cu(SBTH)	15910	45.10
Control	28981	–

Table 4. Percentage inhibition of ^{3}H-uridine incorporation in tumour cells K-562 (human erythroleukemia)

Compound	Dose and inhibition $1\ \mu g\ cm^{-3}$	Inhibition (%) $1\ \mu g\ cm^{-3}$
H_2SBTH	1001	70.51
Cu(SBTH)	1227	63.85
Control	3395	–

compound is not well understood, hence, in order to use this as an antitumour drug, further study is required.

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