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Antitumor Activity Studies of Newly Synthesized *N*-Salicyloyl-*N'*-(*p*-hydroxybenzthioyl)hydrazine and its Copper(II) Complex both in vivo and in vitro

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Abstract—A new ligand *N*-salicyloyl-*N'*-(*p*-hydroxybenzthioyl) hydrazine (H_2STPH) and its Cu^{II} complex [Cu(SPTH)] were prepared and characterized by analytical and physicochemical studies. In vivo antitumor activity of [Cu(STPH)] has been tested against breast tumor in C_3H/J strain mice and in vitro on P-815 (murine mastocytoma) and K-562 (human erythroleukemia) cells. LD_{50} values were estimated at a dose level of 100 mg/kg body weight and a light microscopic study also carried out. © 1997, Elsevier Science Ltd. All rights reserved.

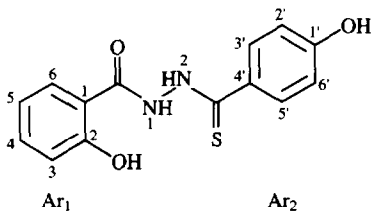
Introduction

It has been established that copper ion chelation plays a definite role in the antineoplastic activity of 3-ethoxy-2-oxobutyraldehyde bis (thiosemicarbozone).[1] In fact, copper(II) complexes of substituted thiosemicarbazones and copper(II) and iron(II) complexes of 5-substitued 2-formylpyridine and 1-formylisoquinoline thiosemicarbazones[2,3] have been found to be cytotoxic to tumor cells in vivo and in vitro. These complexes are strong inhibitors of the enzyme ribonucleotide reductase, an obligatory enzyme in the pathway of synthesis of precursors of DNA.[4–6]

Platinum, iron, copper, palladium and zinc complexes of 2-formylpyridine thiosemicarbazone have been proved to be significant antitumor agents against Ehrlich ascites carcinoma and L1210 leukemia in mice.[7,8]

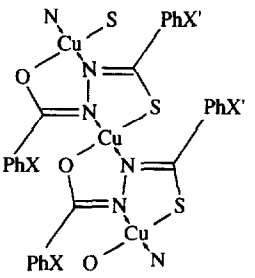
Bis(2-formylpyridine thiosemicarbazonato) copper(II) inhibited the cellular DNA synthesis and DNA isolated from mitochondria, but RNA synthesis was less affected.[9] Copper(II) and cadmium(II) complexes of substituted bis(thiosemicarbazones) inhibited the incorporation of ^{3}H-thymidine into the DNA and respiration of tumor cells.[10,11] Antitumor activity has been reported for some metal chelates of the derivative of dithiocarbazic acid possessing the cytostatic activity in the 9KB test of human epidermoid carcinoma of the nasopharynx[12] and *S*-methyl dithiocarbazate[13] against P388 lymphoid leukemia in mice.

Although thiohydrazides are structurally similar to thiosemicarbazides, not much data is available on the antineoplastic activity of transition metal complexes of thiohydrazides. There is very little information available on the synthesis and structural studies on metal chelates of thiohydrazides and their derivatives.[14–18] Therefore, it was of interest to study the antitumor activity of transition metal complexes of thiohydrazides. *N*-Salicyloyl-*N'*-(*p*-hydroxybenzthioyl)hydrazine (H_2STPH, Fig. 1) and its Cu^{II} complex have, therefore, been synthesized and characterized by analytical and physicochemical methods. The present paper also reports the antitumor activity of H_2STPH and its Cu^{II} complex (Fig. 2) against breast tumor, and in vitro testing against human erythroleukemia cells, and murine mastocytoma cells.



H_2 ST pH

Figure 1.



[Cu (ST PH)], X = OH, X ' = p-OH

Figure 2.

Results and Discussion

The reaction of salicyloyl hydrazine and carboxymethyl *p*-hydroxybenzdithiocarboxylate dissolved respectively in 2 and 1 equiv of aq NaOH resulted in the formation of H_2STPH on addition of dilute CH_3COOH. H_2STPH reacts with Cu^{II} acetate to give a deprotonated complex Cu(STPH), which has a high melting point (>300 °C) and is insoluble in water, ethanol and methanol, but slightly soluble in coordinating solvents such as DMSO and pyridine.

Magnetic properties and electronic spectra

The magnetic moment of 1.85 B.M. for the Cu^{II} complex corresponds to the presence of one unpaired electron. The electronic spectrum of Cu(STPH) shows a broad band at 17540 cm^{-1} assigned to the envelope of $^2B_{1g} \rightarrow {}^2A_{1g}$; $^2B_{2g}$; ^2E_g transtions suggesting a square-planar geometry around Cu^{II}.[19]

ESR spectra

The ESR spectrum of Cu(STPH) at liquid nitrogen temperature shows high-field transition at 3200 G from which $g_\parallel$ and $g_\perp$ have been calculated to be 2.161 and 2.040, respectively.[20] The trend $g_\parallel > g_\perp > 2.0023$ observed for the complex indicates that the unpaired electron is most likely in the $d_{x^2-y^2}$ orbital of Cu^{II}.[19]

IR Spectra

The IR spectrum of H_2STPH in KBr shows two broad bands at 3420 and 3250 cm^{-1} assigned to ν(OH) for the two—OH groups present in the ligand. The first is assigned to the ν(OH) of the salicyloyl part and the second to the *p*-hydroxybenzthioyl part of the ligand. The ν(OH) band due to the salicyloyl part remains unchanged, whereas that due to *p*-hydroxybenzthioyl shows a positive shift of 35 cm^{-1} on complex formation, indicating that both the OH groups of H_2STPH do not take part in complex formation. The positive shift in ν(OH) suggests the breaking up of inter molecular hydrogen bonding on complexation. The bands due to the ν(NH), ν(C=O) and ν(C=S) at 3323, 1626 and 840 cm^{-1} in the ligand are absent in the spectrum of the complex and two new bands appear at ca. 1520 and ca. 700 cm^{-1} assigned to ν(NCO) and ν(C—S) modes, respectively, suggesting the removal of both the —NH— protons via enolization and thio-enolization and bonding of the resulting thiolato sulfur and enolic oxygen with Cu^{II}.[21] The thioamide bands at 1500, 1330 and 1020 cm^{-1} in the spectrum of the ligand due to [β(NH)+ν(CN)], [ν(CN)+β(NH)] and ν(N—N) suffer a positive shift of 50, 40 and 85 cm^{-1}, respectively, in the spectrum of the complex.[22,23] The magnitude of the positive shift in these modes supports the bonding sites indicated above and also suggests the involvement of both the hydrazinic nitrogens in coordination. Thus, H_2STPH behaves as a tetradentate ligand, the bonding sites being the thiol sulfur, enolic oxygen and both the hydrazinic nitrogens.

^1H NMR spectra

The proton NMR spectrum of the ligand exhibits two signals at δ10.13 and δ11.66 ppm for the two —NH—NH— protons adjacent to >C=S and >C=O groups, respectively. The signals at δ3.40 and δ3.46 ppm, are attributed to the —OH protons of *p*-hydroxybenzthioyl and salicyloyl parts, respectively. A multiplet due to benzene ring protons of *p*-hydroxybenzthioyl part appear in the range δ6.8–7.06 ppm, while those of salicyloyl part appear in the range δ7.8–8.04 ppm. The —NH— and —OH proton signals disappear on deuteration of the ligand, thus supporting the above assignments.[24]

The ^{13}C spectrum of the ligand (Fig. 1) shows 11 signals. The assignments for the ^{13}C signals have been made by taking into acount the chemical shift values of *p*-hydroxyphenylthiocarboxyhydrazide (C=S 183.5, C_1 159.7, C_4 129.7, $C_{3,5}$ 129.2, $C_{2,6}$ 114.80) and salicyloyl hydrazone.[25] In view of the paramagnetic nature and insufficient solubility of Cu(STPH) in DMSO-d_6 it was not possible to record its ^1H and ^{13}C NMR spectra.

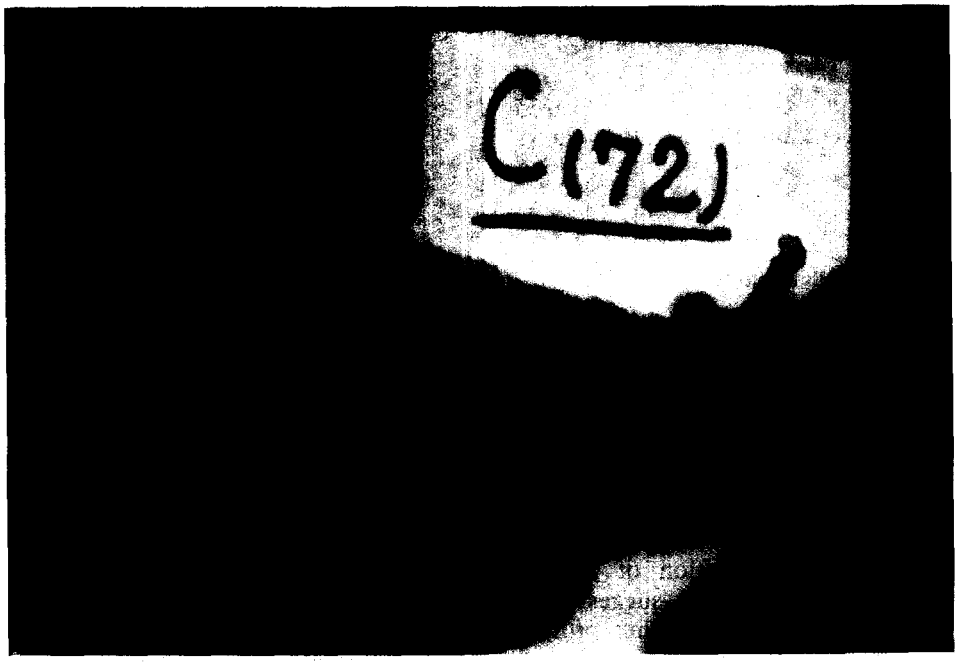
Antitumor screening

The results obtained in this study reveal that the compound is effective against experimental breast tumor when the dose level of 10 mg/kg body weight (i.p.) was given and was more effective when the dose level was just doubled, i.e., 20 mg/kg body weight (i.p.). The average body weight change was 2.33–5.51 g and the gross weight reduction in the tumor was 25–35% for curative and preventive, respectively. The life span of treated animals increased significantly in comparison to control and shows pronounced antitumor activity with *T/C* values of 225.0% for preventive and 175.71% for curative. It was also seen that the untreated and controls died earlier, but more than 50% of the treated animals survived after 3 months in case of curative studies with [Cu(STPH)]. In the initial state of transplantation the tumor growth was retarded by this complex. Sometimes the tumor becomes converted into wound and wound into pus and pus comes out from the body.

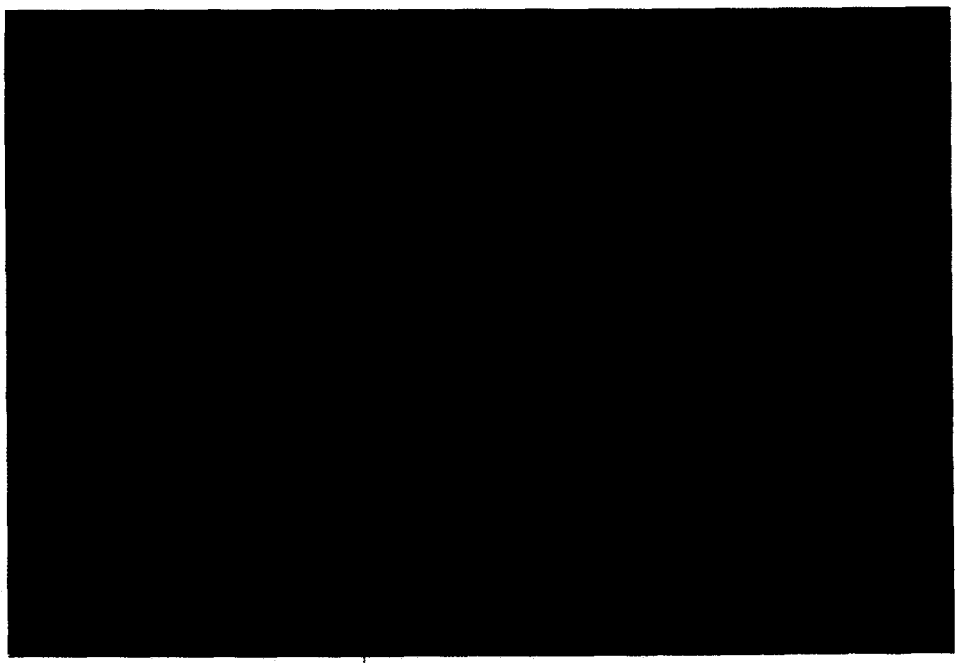
A light microscopic study reveals that untreated breast tumor shows compact cellular organization in which the cells have an oval-shaped large nucleus with a single prominent nucleus. A large number of mitotic figures can be seen in an untreated mass of tumor (photograph C). Whereas, following the injection of the test compound, the cellular compactness is lost (photograph D). The cytoplasm appears to be distorted, the nucleus appears more transluscent with chromatin dispersed in the form of granules, and finally the cytoplasmic boundaries are disrupted. One can observe the infiltration of a large number of leukocytes, lymphocytes and macrophages in the regressing tumor mass after treatment. The antitumor potential activity of *cis*-dichlorodiammine platinum (II) (cisplatin), which has also been shown to be effective against some of the transplantable tumors such as

sarcome 180 in C_3H/HeJ strain mice regressed completely after a single i.p. injection of 10 mg/kg body weight without any apparent irreversible damage to the host, and also upon cisplatin treatment, the mitotic activity was immediately inhibited and there was increased infiltration of lymphocytes and macrophages in the regression of tumor mass.[26,27]

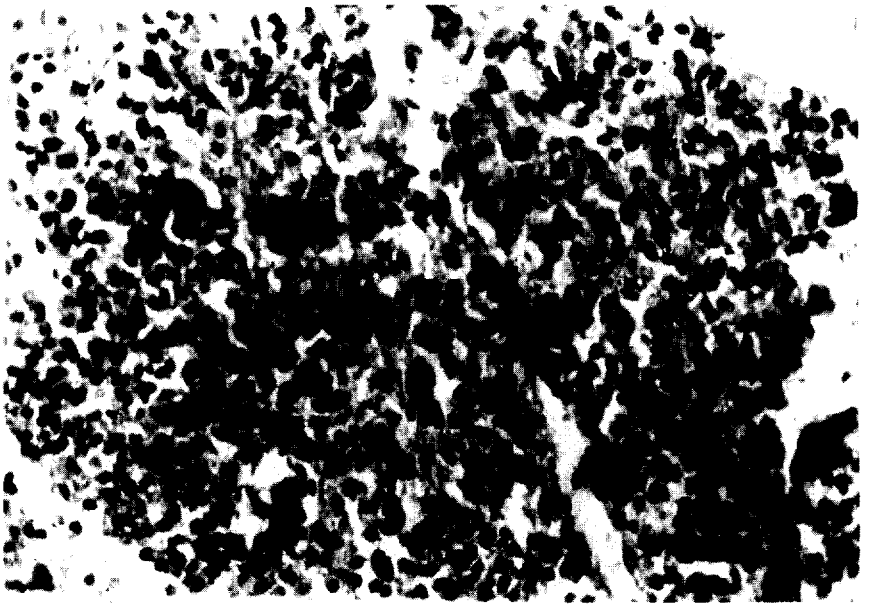
Earlier reports from our laboratory suggest that *N*-salicyloyl-*N'*-(2-furylthiocarbonyl)hydrazine (H_2SFTH), Cu(SFTH) and (*N*-salicyloyl-*N'*-benzthioylhydrazinato) copper(II) abbreviated as Cu(SBTH) show *T/C* values of 195, 165 (for Dalton's lymphoma)[17] and 193 (for breast tumour cells)[18] respectively. A *T/C* values of more than 125 indicates that the complex is worthy of testing in other tumour systems. We also tested the compounds for inhibitory effect on ^{3}H-thymidine and ^{3}H-uridine incorporation in K-562 human tumor cell line and P-815 murine tumor cell line, in vitro at 1 mg/mL and 5 mg/mL doses (Table 1–4). There was



Photograph A. Untreated female mouse (control).



Photograph B. Treated female mouse.



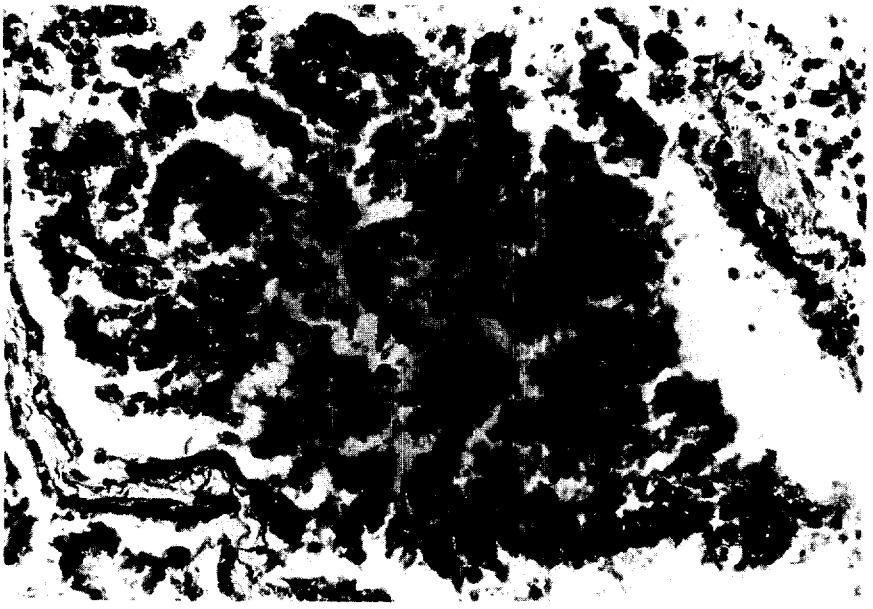
Photograph C. Untreated tumor cells (control).

significant inhibition of ^{3}H-thymidine or ^{3}H-uridine incorporation in both K-562 as well as P-815 tumor cells. The compounds at the specified dose were not directly cytotoxic to the tumour cells but they had a cytostatic effect. As shown in the tables, inhibition of thymidine incorporation (Tables 1 and 3) both in the case of K-562 and P-815 tumour cells suggests inhibition of cell proliferation and inhibition of uridine incorporation (Tables 2 and 4), which suggest that RNA synthesis is checked thereby affecting protein synthesis and the general metabolic activity of tumour cells. The effect is more pronounced in the case of murine tumor cells at lower dose. Both in vitro and in vivo results obtained in the present study showed that this compound has strong antitumor potential. The mechanism of antitumor action of this compound is not well understood, hence, in order to use this as a potential antitumor drug more studies are needed.

Experimental

Preparation of H_2STPH

Salicylic acid hydrazide[28] and carboxymethyl *p*-hydroxybenzdithiocarboxylate[29] were prepared by the literature methods. Equimolar amounts of salicylic acid hydrazide and carboxymethyl *p*-hydroxybenzdithiocarboxylate were dissolved separately in 2 and 1 equiv of aq 2 N NaOH, respectively, the solutions were



Photograph D. Treated tumor cells.

Table 1. Percentage inhibition of ^{3}H-thymidine incorporation in tumor cell (P-185) murine mastocytoma

Compound	Dose and inhibition counts/min		% Inhibition	
	1 μg/mL	5 μg/mL	1 μg/mL	5 μg/mL
H_2 (STPH)	7359	12,479	50.81	16.60
Cu (STPH)	7210	10,109	51.81	32.44
Control	14,963			

mixed, and the mixture was left for ~2 h at room temperature. The desired product was precipitated by adding dilute acetic acid dropwise to the above reaction mixture. The product thus obtained was suction filtered, washed with water, dried and recrystallized from hot ethanol (mp 158–160 °C). analyses ($C_{14}H_{12}O_3N_2S$): C, H, N, S calcd 58.33, found 57.49; calcd 4.16, found 3.68; calcd 9.72; found 8.18; calcd 11.11; found 10.16, respectively. IR (cm^{-1}): ν(OH), 3420 and 3250; ν(NH), 3323; ν(C=O), 1626; ν(C=S), 840. ^{1}H NMR (δ, ppm): NH, 10.13 and 11.66; OH, 3.40 and 3.46; ring protons, 6.8–7.06 and 7.8–8.04. ^{13}C NMR (δ, ppm): C=S, 190.32; C=O, 163.51; C_1 115.72; C_2 157.49; C_3, C_5 114.69; C_4 129.07; C_6 119.57; C'_1 160.63; C'_2, C'_6 117.02; C'_3, C'_5 129.97; C'_4 134.03.

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

Compound	Dose and inhibition counts/min		% Inhibition	
	1 μg/mL	5 μg/mL	1 μg/mL	5 μg/mL
H_2 (STPH)	26,258	20,405	51.23	62.10
Cu (STPH)	11,132	16,290	79.32	69.74
Control	53,842			

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

Compound	Dose and inhibition counts/min	% Inhibition
	1 μg/mL	1 μg/mL
H_2 (STPH)	20,033	30.87%
Cu (STPH)	17,750	38.75%
Control	28,981	

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

Compound	Dose and inhibition counts/min	% Inhibition
	1 μg/mL	1 μg/mL
H_2 (STPH)	7359	50.81
Cu (STPH)	7210	51.81
Control	14,963	

Preparation of Cu(STPH)

The complex was prepared by digesting the solution of copper(II) acetate monohydrate (1.99 g in 60 mL of 50% methanol) and the ligand (2.72 g in 10 mL of methanol +1 g of sodium acetate) in a boiling water bath for ~15 min. On cooling, the light brown complex thus obtained was suction filtered, washed successively with a water:methanol mixture (50:50 v/v) and hot ethanol, and dried in vacuo. The complex was analyzed for its metal, nitrogen and sulfur contents following the standard procedures as reported earlier.[30] Analysis $Cu(C_{14}H_{10}N_2O_3S)$: Cu, C, H, N, S : calcd 18.17, found 18.0; calcd 48.06, found 47.67; calcd 2.86, found 2.74; calcd 8.01, found 7.70; calcd 9.15, found 9.01, respectively.

Physical measurements

The complex was analysed for its copper content employing a standard procedure after decomposition with a mixture of HNO_3 and HCl followed by H_2SO_4. Sulfur was estimated as $BaSO_4$. C, H and N were analysed on a Perkin–Elmer C, H, N analyser, model 240 C.

Magnetic susceptibilities, and electronic and IR spectra were obtained as described earlier.[31] The X-band ESR spectrum was obtained in DMSO on a EPR-E-112 spectrometer using DPPH as a $<g>$marker. ^{1}H and ^{13}C NMR 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 complete medium (RPMI 1640 medium supplemented with antibiotics, penicillin, streptomycin and 10% heat inactivated fetal calf serum) at a concentration of 10^6 cells/mL following the literature method.[32] 2×10^5 cells/well 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/mL) and incubated for 24 h at 37 °C in a CO_2 incubator. In the 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 ($400 \times g$ for 10 min). The cell pellets were resuspended in 0.2 mL complete medium containing 1 μCi/mL ^{3}H-thymidine or ^{3}H-uridine and pulse labeled for 4 h for thymidine and 2 h for uridine. The cells were then washed thrice with PBS (phosphate-buffered saline). 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:

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

In vivo studies

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

The therapeutic effectiveness of the compound against tumor-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 tumor free survivors.

Mammary gland tumor system for evaluation of antitumor effect

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

Test for toxicity

Animals were transplanted the cells of mammary gland tumor subcutaneously near the nipples by making air pockets. On day three of transplantation of mammary gland tumor, the test compound in different doses was injected i.p. as a single injection; five animals were used per dose level. Toxic doses or LD_{50} values were estimated on the basis of survivals on the fifth day of injection and found to be 100 mg/kg body weight of the animal. The CNS studies suggest that the drug is remarkably safe, having no adverse effect on any of the other parameters studied. However, there was only slight reduction in aggressive behavior and response to stimuli in the higher dose range (100 mg/kg) in 1–2 h. The drug shows marked central behavioral effect and a slight CNS depressant effect. The compound has analgesic effect but no sedative effect. At higher dose (100 mg/kg) only reduction of aggression was observed without affecting any other parameter.

Histological study

Animals from both control and treated batches were killed at 2 day intervals up to 6 days. Tumor tissue with liver, kidney, lung, heart and spleen was fixed in Bouin's fluid (aq), dehydrated, kept in cedar wood oil for 3 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 tumor cells, Harries's hemotoxylin eosin stain was used and other processes were same.

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