Research Paper

Cytotoxic and Antitumour Studies of Acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone and its Transition Metal Complexes

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Cytotoxic activities of acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone (L2H) and its seven different metal complexes were studied. Of these, IC_{50} value of the copper complex was found to be 46 μg/ml. Antitumour studies of this copper complex was carried out using Daltons Lymphoma Ascites cell-induced solid tumour model and Ehrlich's Ascites Carcinoma cell-induced ascites tumour model. Administration of the copper complex at different concentrations (10, 5 and 1 mg/kg b. wt) inhibited the solid tumour development in mice and increased the mean survival rate and the life span of Ascites tumour bearing mice in a concentration dependent manner.

Key words: Daltons lymphoma ascites, Ehrlich ascites carcinoma, cytotoxicity, ligand, acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone

Coordination compounds have immense applications in different fields such as metallurgy, industry, biology and medicine. Although metals are used in the medical field, the actual role and potential of metal based drugs were fully understood only after the discovery of cisplatin, i.e., cis-[dichlorodiammine] platinum (II)[1]. Cisplatin and carboplatin still play prominent role in cancer chemotherapy[2,3].

Since the discovery of the antitubercular activity of thiosemicarbazones[4], a great deal of work has been done on the pharmacology of this type of compounds. Literature survey revealed that the transition metal complexes of thiosemicarbazones show antitumour property[5]. Thiosemicarbazones exercise their biological activity in mamalian cells by inhibiting ribonucleotide reductase, a necessary enzyme in the synthesis of DNA precursors[6]. Studies on iron and copper complexes have shown that they can be more active in the inhibition of DNA synthesis than the free thiosemicarbazone[7]. Previous studies showed that the copper(II) complex of 2-formylpyridine thiosemicarbazone is more powerful antitumour agent than the free ligand[8]. The antitumour activities of Co(II), Ni(II), Cu(II) and Zn(II) complexes of thiosemicarbazone derived from 3-acetylumbelliferone was studied[9]. It was confirmed that the Co(II) and Cu(II) complexes produced more inhibitory effect compared to the other complexes.

Since the cost of platinum complexes is very high, complexes of cheaper metals like copper and iron were tried in cancer chemotherapy. According to Petering[10], Cu(II) ion alone will not have any antitumour activity, but will act as an inhibitor of tumour growth in the chelated form. Even though, the actual pathway by which the copper chelate inhibits the cancer growth is not known, it is believed that this will be on the basis of structure activity correlation as in the case of cisplatin[11] (i.e. DNA intercalation). Earlier studies based on the inhibition of DNA biosynthesis showed that copper complexes

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were more active as antitumour agents than those of Fe(III), probably due to the reversible reduction processes at accessible electrode potential of copper compounds[12]. Secondly, 4-coordinate planar copper complexes will get attached to nitrogen base of DNA, thus blocking the base replication leading to the inhibition of tumour growth than 6-coordinate complexes of Fe(III)[13].

Among the important mechanistic conclusions emanated from the structural studies, a prominent one is the finding that the ligand systems with NNS, ONO or ONS donor atoms have ample carcinostatic potency. Recently, it has been shown that substitution on the 4th nitrogen of thiosemicarbazone can enhance its biological activity[14]. Our literature survey revealed that semicarbazone, thiosemicarbazone and related compounds of 1,3-diketoderivatives have been seldom investigated for their biological activities[15,16]. Hence, it is considered to be worthwhile and interesting to evaluate the antitumour activity of acetoacetanilide N-(4)methyl(phenyl) thiosemicarbazone (AcTSC) and its metal complexes.

MATERIALS AND METHODS

All chemicals used were of AnalaR quality and purchased from Merck. Commercial solvents used for the synthesis were purified by standard methods[17]. Hydrated metal salts purchased were used as such for the preparation of the complexes.

Preparation of the ligand (acetoacetanilide N(4)-methyl(phenyl)thiosemi-carbazone):
Acetoacetanilide (0.005 mol) in 30 ml methanol was added to N(4)-methyl(phenyl)thiosemicarbazide (0.005 mol) in 30 ml methanol and refluxed on a water bath for 3 h. The mixture was cooled to room temperature. The solid product obtained was filtered, dried and kept over fused calcium chloride (Yield: 70%, M.P=158º)[18].

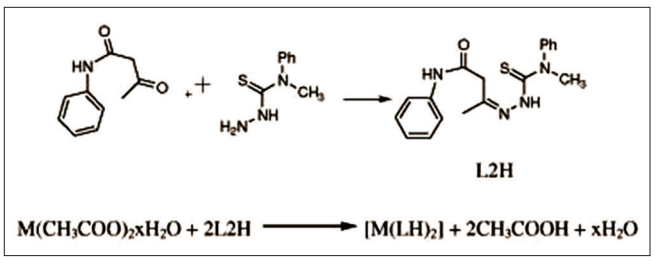
Preparation of the thiosemicarbazone complexes:
A methanol solutions of metal acetate (0.025 mol in 20 ml) was added to a methanol solution of AcTSC (0.05 mole in 40 ml) and the mixture was refluxed for 4 h. Then it was evaporated and cooled. The solid complex that formed was filtered off, washed several times with methanol and water. It was dried in a desiccator. The product was kept over fused calcium chloride. The complexes of Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) were prepared using a reaction mixture containing metal acetate and ligand in 1:2 molar ratio. Yield and melting point of complexes were noted (Yield: 70–80%, m.p.=210–220º)[18] (Scheme 1).

Characterization of the ligand and complexes:
The ligand (fig. 1a) and the complexes (fig. 1b) were characterized by elemental analysis, magnetic moment measurements, IR, UV/Vis spectrum and ^{1}H NMR spectral studies[18].

The preparation of the drug:
Fifty milligrams of the compound was dissolved in 1 ml of dimethyl sulphoxide (DMSO). For *in vitro* studies, the drug was dissolved in DMSO and for *in vivo* studies 50 mg of drug was first dissolved in 1 ml DMSO and further it was diluted using distilled water to desired concentration.

Evaluation of anticancer potential:
Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines were initially procured from Adayar Cancer Institute, Chennai and propagated as transplantable tumors in the peritoneal cavity of BALB/C mice. L929 (mouse lung fibro blast) cell line was obtained from National Centre for Cell Sciences, Pune.

Swiss albino female mice (20-25 g) were obtained from the Small Animal Breeding Station (SABS), Mannuthy, Thrissur, Kerala. They were kept under standard conditions of temperature and humidity in animal house of Amala Cancer Research Centre. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All the animal experiments in this



Scheme 1: Synthetic scheme of preparation of the ligand and the complexes.
M is Fe(II), Co(II), Ni(II), Cu(II), Zn(II), or Cd(II) and LH is acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone.

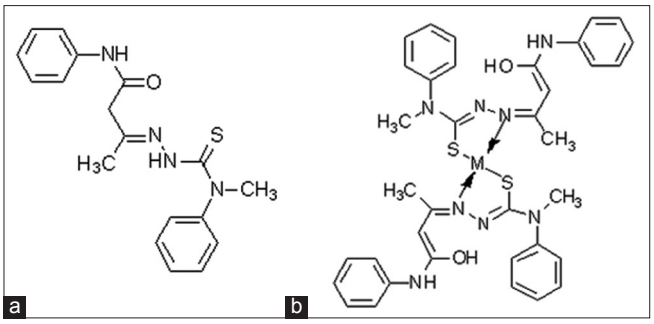


Fig. 1: Structures of acetoacetanilide N(4)-methyl(phenyl) thiosemicarbazone and its complexes.
Structure of (a) acetoacetanilide N(4)-methyl(phenyl) thiosemicarbazone and (b) structure of metal complex of acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone.

study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Mouse lung fibroblast (L929 cells) were cultured in DMEM medium supplemented with FBS (10% v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml) and kept at 37° in an incubator with 5% CO_2. Dalton's Lymphona Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cells maintained in the intraperitoneal cavity of mouse, were used for the study.

Trypan blue exclusion method:
The test compounds were studied for short-term *in vitro* cytotoxicity using Dalton's lymphoma ascites cells (DLA). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by Trypan blue exclusion method[18]. Viable cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 h at 37°. Further cell suspension was mixed with 0.1 ml of 1% Trypan blue and kept for 2–3 min and loaded on a haemocytometer. Dead cells take up the blue colour of Trypan blue while live cells do not take up the dye. The stained and unstained cells were counted separately.

Methyl tetrazolium assay:
For long-term cytotoxicity, L929 cells were used. The cells were seeded in to 96 well flat bottom titre plate (5000 cells/well) containing 200 µl MEM (minimum essential medium) with 10% FCS (fetal calf serum) and incubated for 24 h at 37° in 5% CO_2 atmosphere for the attachment of cells. After incubation, various concentrations of the test compound were added to the wells in triplicates and the incubation was continued for 48 h. Twenty microlitres of MTT (5 mg/mL in PBS) was added to each well before 4 h of the completion of incubation. After the incubation period, the plates were centrifuged, supernatant liquid was removed and 100 µl of DMSO was added to each well. The plate was then incubated at room temperature for 15 min and the optical density (OD) was measured at 540 nm. The percentage of dead cells was determined using the formula: % of dead cells=(I–OD of drug treated/OD of control)×100.

Toxicity studies of metal complexes:
Twenty four Swiss albino mice were divided into 4 groups (6 animals/group). Group 1: 1 mg/kg, treated, Group 2: 5 mg/kg, treated, group 3: 10 mg/kg, treated and group 4: 25 mg/kg, treated. The drug was administrated once daily (i.p.) and continued for 10 weeks. The animals were observed for their mortality.

Effect of copper complex of acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone on the survival rate of ascites tumour bearing animals:
Animals (female, 6–8 weeks old) weighing 28–30 g were divided into 4 groups of 6 animals each. Viable EAC cells 10^6 in 0.1 ml of phosphate buffered saline (PBS) were injected in to the peritoneal cavity. Group 1: control, group 2: 1 mg/kg, treated, group 3: 5 mg/kg, treated, group 4: 10 mg/kg, treated, and group 5: standard drug (cyclophosphamide), treated.

Drug and cyclophosphamide were given by intraperitoneal injection from the first day of tumour induction. The death pattern of animals due to tumour burden was noted and the percentage increase in life span was calculated as, %ILS=(T-C/C)×100, where T and C are mean survival of treated and control mice, respectively.

Effect of copper complex of acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone on solid tumour development:

Swiss albino mice (female, 5–6 w old) weighing 20–25 g were divided into 4 groups comprising of 6 animals in each group for the above studies. Tumour was induced by injecting DLA cells (0.1 ml of 10^6 cells per mouse) in to the right hind leg of mice. Group 1 was kept as control Groups 2, 3 and 4 were treated with copper complex of AcTSC. Group 5 was treated with cyclophosphamide. The tumour development on animals of each group was determined by measuring the diameter of tumour growth in two perpendicular planes using a digital vernier caliper starting from 7th day of tumour induction up to 34th day. The tumour volume was calculated using the formula, $V=4/3\pi r_1^2 r_2$, where r_1 is the minor diameter and r_2 is the major diameter[19].

Statistical analysis:

The values were expressed as mean±standard deviation (SD). The mean values were statistically analyzed using one-way analysis of variance (ANOVA) using graph pad Instat 3 software (Graph Pad Software, San Diego, California, USA) followed by appropriate *post-hoc* test (Dunnett multiple comparison test). $P<0.05$ was considered statistically significant and are indicated by "*".

RESULTS

Short-term *in vitro* cytotoxic analysis:

The ligand, AcTSC and its Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) complexes showed marked cytotoxic activity for DLA cell line (Table 1). The copper complex showed maximum activity (fig. 2) and the concentration required for 50% death (IC_{50}) was found to be 46 µg/ml. The free ligand showed only very low cytotoxic activity.

TABLE 1: *IN VITRO* CYTOTOXICITY OF AcTSC AND ITS DIFFERENT COMPLEXES

Concentration (µg/ml)	Percentage cytotoxicity						
	Complexes						Ligand
	Fe	Co	Ni	Cu	Zn	Cd	AcTSC
10	0	0	3	18	7	2	0
20	2	0	7	24	14	9	0
50	6	5	11	58	20	12	0
100	10	8	20	70	35	36	2
200	20	18	33	92	58	42	4

AcTSC: Acetoacetanilide N-(4)-methyl(phenyl)thiosemicarbazone

Long-term *in vitro* cytotoxic analysis (methyl tetrazolium assay):

The results of long-term *in vitro* cytotoxicity of the copper complex of AcTSC showed that it is nontoxic up to 0.5 µg/ml towards L929 cells (Table 2).

Toxicity studies:

The results of toxicity studies of copper complex on 24 Swiss albino mice, 4 groups, at four concentrations (25, 10, 5 and 1 mg/kg) showed that 25 mg/kg was toxic to the animals. Therefore, this concentration was avoided and 10, 5 and 1 mg/kg were only selected for *in vivo* studies, as they were nontoxic to the animals.

Effect of copper complex on ascites tumour development:

The animals of the tumour control group survived for a period of 15.8±0.65 d. Those treated with cyclophosphamide survived for 26.63±1.8 d. The copper complex at 10, 5 and 1 mg/kg increased the survival rate of animals by 21±2.1 d, 19.6±1.4 d, and 18.6±1.2 d, respectively (Table 3). Thus the copper complex was found to be effective in increasing the average life span of the animals by 32.9, 24.05 and 17.7%, respectively, at 10, 5 and 1 mg/kg doses (Table 4).

Effect of copper complex on solid tumour development:

In the control animals, the volume of tumour was increased to 2.973 cm^3 on 34th d, while in the animals treated with the copper complex, there was a significant reduction of tumour volume. At 10 mg/kg, the volume was 1.1605 cm^3, while at lower

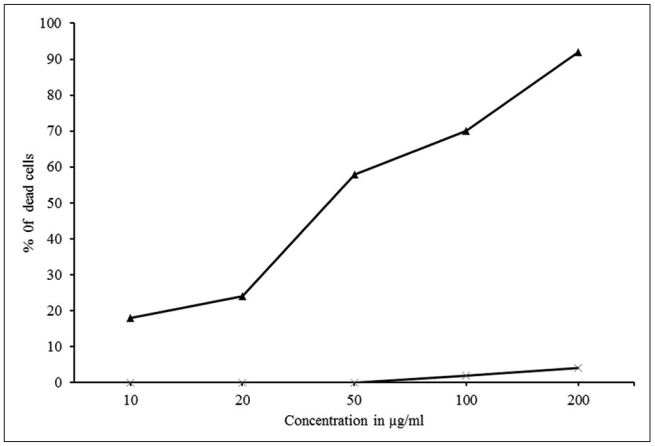


Fig. 2: Cytotoxic action of acetoacetanilide N-(4)-methyl(phenyl) thiosemicarbazone and its copper complex.

concentrations (5 and 1 mg/kg) the tumour volumes were 1.4692 and 2.1011 cm^3, respectively. Treatment with cyclophosphamide, a standard antitumour drug reduced the tumour volume to 0.583 cm^3 (Table 5 and fig. 3).

DISCUSSION

In 2008, approximately 12.7 million cancer cases were diagnosed and 7.6 million people died of cancer. The most common, being the cancer that affecting stomach, lung, liver and breast. Though many diseases (such as heart failure) may have worse effect on the society, it is believed that cancer is the most dangerous and deadly disease affecting human being. Therefore, cancer is regarded as a disease that must be "fought" to end. Here lies the importance of cancer research, which includes the intense scientific efforts to understand the disease processes and to discover the possible therapies.

Bearing in mind that the N-N-S or O-N-O or O-N-S donor system is a common feature for all compounds with carcinostatic potency, we proceeded the antitumour studies of acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone and its metal complexes and we got promising results. *In vitro* cytotoxicity studies on AcTSC and its different complexes showed cytotoxicity against DLA cell lines. The copper complex showed maximum cytotoxicity with an IC_{50} value of 46 μg/ml.

Ehrlich ascetic tumor is a rapidly growing carcinoma with very aggressive behavior[20]. It is able to grow in almost all strains of mice. The Ehrlich ascetic tumor implantation induces a local inflammatory reaction with increasing vascular permeability, which results

TABLE 2: MTT ASSAY OF COPPER COMPLEX OF AcTSC

Concentration (μg/ml)	Cytotoxicity (%)
Control	0
0.05	0.37±0.03
0.1	0.51±0.03
0.25	0.61±0.03
0.5	1.05±0.03

AcTSC: Acetoacetanilide N-(4)-methyl(phenyl)thiosemicarbazone, MTT: methyl tetrazolium

TABLE 3: EFFECT OF COPPER COMPLEX OF AcTSC ON THE MEAN SURVIVAL RATE OF ASCITES TUMOR BEARING MICE

Treatment (mg/kg)	Mean survival rate
Control	15.8±0.65
10	21±2.1
5	19.6±1.4
1.5	18.6±1.2
Standard-cyclophosphamide (10)	26.63±1.8

AcTSC: Acetoacetanilide N-(4)-methyl(phenyl)thiosemicarbazone

TABLE 4: EFFECT OF COPPER COMPLEX OF AcTSC ON THE LIFE SPAN OF ASCITES TUMOUR BEARING MICE

Treatment (mg/kg)	Increase in life span (%)
Control	-
10	32.9
5	24.05
1	17.7
Standard-cyclophosphamide (10)	68.4

AcTSC: Acetoacetanilide N-(4)-methyl(phenyl)thiosemicarbazone

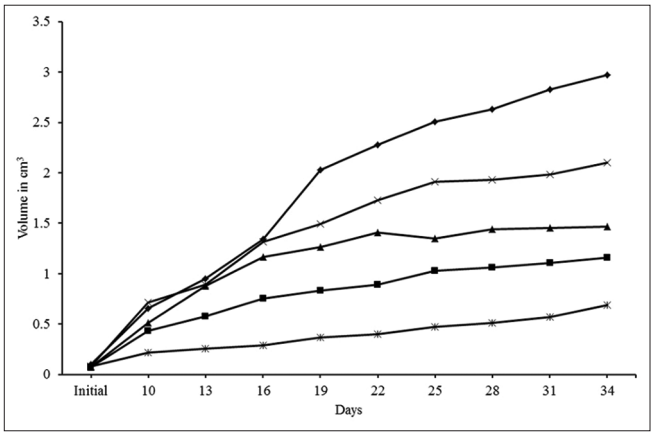


Fig. 3: Effect of copper complex of AcTSC on soild tumour induced by Dalton's lymphoma ascites cells.
The line graph for control (-♦-), 10 mg/kg (-■-), 5 mg/kg (-▲-), 1 mg/kg (-×-), standard: cyclophosphamide-10 mg/kg (-*-) is constructed for tumour volume versus number of days.

TABLE 5: EFFECT OF COPPER COMPLEX OF AcTSC ON THE REDUCTION OF TUMOUR VOLUME

Treatment (mg/kg)	Observation (number of days)									
	Initial	10	13	16	19	22	25	28	31	34
Mean volume	0.099	0.659	0.835	1.344	2.035	2.283	2.514	2.636	2.83	2.973
10	0.075	0.436	0.576	0.756	0.834	0.889	1.032	1.059	1.11	1.161
5	0.08	0.511	0.877	1.168	1.263	1.408	1.351	1.440	1.46	1.469
1	0.083	0.714	0.885	1.314	1.495	1.777	1.912	1.929	1.98	2.101
Standard-cyclophosphamide (10)	0.080	0.220	0.260	0.290	0.370	0.400	0.470	0.510	1.57	0.690

Values are in cm^3. AcTSC: Acetoacetanilide N-(4)-methyl(phenyl)thiosemicarbazone

in an intense edema formation, cellular migration and a progressive acetic fluid formation[21]. The ascetic fluid is essential for tumor growth since it constitutes a direct nutritional source for tumor cells[22]. The copper complex of AcTSC was found to be effective against DLA-induced solid tumour and EAC-induced ascites tumour. The 10 mg/kg body weight was more effective than the other two concentrations (5 and 1 mg/kg b.wt) in both the cases. The present study of *in vitro* cytotoxic and antitumour properties of the copper complex of acetoacetanilide N(4)-methyl(phenyl)thiosemicarbazone (AcTSC) suggests its potential use as an anticancer agent.

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Conflicts of interest:
There are no conflicts of interest.

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