Part IV
Antitumour studies

Chapter 2

Nimmy Kuriakose “Physicochemical, thermoanalytical, electrochemical and antitumour studies of transition metal complexes of schiff bases derived from heterocyclic carbonyl compounds” Thesis. Department of Chemistry, St. Thomas College, University of Calicut, 2015

CHAPTER 2

MATERIALS AND METHODS

Some of the newly synthesized copper complexes were taken for antitumour studies since a variety of Schiff bases and their copper complexes were reported to have marked antitumour activity. The activities of the complexes were studied at Amala Cancer Research Centre, Thrissur, Kerala. Firstly the copper complexes of different ligands were subjected to *in vitro* cytotoxic activity studies and then highly active complexes were selected for conducting *in vivo* antitumour studies.

Drug

Cu(II) complexes of different Schiff base ligands, 3-(1H-indol-3-yl)-2-[(thiophen -2-ylmethylidene)amino]propanoic acid (I3YT2YMAPA), 3-[thiophen-2-ylmethylene amino]benzoic acid (T2YMABA), 4-(5-[(2-carbamothioylhydrazono)methyl]thiophen-2-yl)benzoic acid (CTHMT2YBA), 4-(5-[(2-phenylhydrazono)methyl]thiophen-2-yl) benzoic acid (PHMT2YBA), 4-(5-[(2-carbamothioylhydrazono)methyl]furan-2-yl) benzoic acid (CTHMF2YBA), 2-(1-[pyridin-3-yl]ethylidene)hydrazinecarbothioamide (P3YEHCTA), 3-(1-(2-phenyl hydrazono)ethyl)pyridine (PHEP), 3-[anthracen-9(10H)-ylideneamino]propanoic acid (A9Y3APA), 2-[anthracen-9(10H)-ylideneamino]-3-(1H–imidazole–4-yl)propanoic acid (A9Y3IMPA) and 2-[anthracen-9(10H)-ylideneamino]-3-phenyl propanoic acid (A9Y3PPA) are screened for their antitumour activity.

The first five Cu(II) complexes were synthesized and characterised as described in Part I. The remaining complexes were prepared according to the procedure given in published works[52,53]. The structures of these complexes and the standard drug cyclophosphamide are given in Figure 4.2. All the chemicals were of analar quality and

purchased from E. Merck. Commercial solvents used for the synthesis were purified by standard methods.

Animals

Swiss albino male mice (20-25g) were obtained from the Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala. They were kept under standard conditions of temperature and humidity in well ventilated cages in the animal house of Amala Cancer Research Centre, Thrissur. The animals were provided with standard mouse chow (Sai Durga). Studies were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and 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.

Cell lines

Dalton's lymphoma ascites cells (DLA), maintained in the intraperitoneal cavity of mouse, were used for the study**.** The ascites fluid was removed by aspiration and the cells were sedimented by centrifugation for 10 minutes. The cells were further washed with Phosphate Buffered Saline (PBS) twice and were suspended uniformly. The numbers of cells were assessed by haemocytometer. One million cells in 0.1ml of PBS (pH = 7.2) were used for each experiment**.**

Short term *in vitro* cytotoxic analysis

Materials required

1. Dalton's lymphoma ascites cells
2. Phosphate Buffered Saline (PBS) : 500ml solution contains

- NaCl - 4g

- Na_2HPO_4 - 0.72g
- KH_2PO_4 - 0.1g
- KCl - 0.1g

3. Trypan blue solution
4. Haemocytometer.
5. Pipette (1ml, 500µl, 100µl capacity)
6. Test tubes (10ml)
7. Microscope
8. Dimethyl sulphoxide (DMSO)

Preparation of the drug

The drug was prepared by dissolving ten milligrams of each copper(II) complexes in 1ml dimethyl sulphoxide (DMSO), which is a non-toxic solvent compared to other organic solvents. From this stock solution, using a micropipette, five different concentrations i.e., 200µg/ml, 100µg/ml, 50µg/ml, 20µg/ml and 10µg/ml were prepared with Phosphate Buffered Saline solution.

Trypan blue exclusion method

Cu(II) complexes of Schiff bases were studied for short term *in vitro* cytotoxicity using Dalton's lymphoma ascites (DLA) cells. The tumour cells were aspirated from the peritoneal cavity of tumour bearing mice. The cells were washed thrice with Phosphate Buffered Saline or normal saline. Trypan blue exclusion method was followed to determine the cell viability**.**

Ten copper complexes of different ligands were taken for cytotoxic study. Five different concentrations (200µg/ml, 100µg/ml, 50µg/ml, 20µg/ml and 10µg/ml) of each

complex were prepared with Phosphate Buffered Saline (PBS) solution in test tubes. Viable cell suspension ($1x10^6$ cells in 0.1ml) was added to tubes containing various concentrations of the test compounds and the volume was made upto 1ml using Phosphate Buffered Saline(PBS). Two control tubes containing only the cell suspension were also kept. These assay mixture were incubated for 3 hours at 37^oC. Further cell suspension was mixed with 0.1 ml of 1% trypan blue, kept for 2-3 minutes and loaded on a microscope connected with haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye and they have the typical shiny appearance. The numbers of stained and unstained cells were counted separately through the microscope using haemocytometer. Then the effect of various concentrations of the samples to produce cytotoxicity was calculated as,

$$\% \text{ cytotoxicity} = \frac{\text{Number of dead cells}}{\text{Number of dead cells + number of live cells}} \times 100$$

The Schiff base ligands were also subjected to *in vitro* cytotoxicity studies to ascertain their activity in comparison with their copper complexes.

Toxicity studies of Schiff base complexes

Materials required

1. Swiss albino mice (male) -12 numbers
2. Dimethyl sulphoxide (DMSO)
3. Drug solution
4. Injection syringe (1ml)
5. Test tubes (10ml)

Preparation of the drug

Three different concentrations of the drug were prepared as follows.

1. 20mg/kg b.wt. : 4mg of the Cu(II) complex was dissolved in 100µl DMSO and made upto 2ml with distilled water.
2. 10mg/kg b.wt. : 4mg of the Cu(II) complex was dissolved in 100µl DMSO and made upto 4ml with distilled water.
3. 2mg/kg b.wt. : 4mg of the Cu(II) complex was dissolved in 100µl DMSO and made upto 20ml with distilled water.

Toxicity studies

Swiss albino mice, weighing 20-25g were taken for conducting the toxicity studies of the complexes. In order to find out the nontoxic concentration, the mice were divided into 4 groups (4 animals/group). The drug was administrated to each group as follows:

Group 1: control (untreated)

Group 2: 20mg/kg b.wt, treated,

Group 3: 10mg/kg b.wt, treated,

Group 4: 2mg/kg b.wt, treated.

The drug (200µl) was given as injection to intraperitoneal cavity of each animal once on alternate days and continued for five days. They were kept under standard conditions of temperature and humidity in well ventilated cages at animal house of Amala Cancer Research Centre, Thrissur. The animals were provided with standard mouse chow and water. The animals were observed for their mortality each day. From the death rate of the mice, the nontoxic concentration was determined.

***In vivo* ascites tumour reduction studies**

Materials required

1. Swiss albino mice (male) weighing about 25g - 48 numbers
2. Dimethyl sulphoxide (DMSO)
3. Drug [Cu(II) complexes]
4. Cyclophosphamide solution
5. Injection syringe (1ml)
6. Distilled water

Preparation of the drug

2mg/kg b.wt concentration solutions of the drugs were prepared first, as described earlier. Then it was properly diluted using distilled water in order to get solutions of the concentrations 0.5mg/kg b.wt and 0.25mg/kg b.wt. These two concentrations were employed for the *in vivo* tumour reduction studies.

Preparation of the standard drug **(**Cyclophosphamide)

The standard drug solution was prepared by dissolving 20mg cyclophosphamide in 3.2ml distilled water to form 25mg/kg b.wt solution.

Ascites tumour reduction studies

The effect of copper complexes on the survival rate of ascites tumour bearing cells was noted as follows.

Animals weighing 20-25g were divided into four groups (6 animals/group) as follows:

Group 1: Control group (untreated)

Group 2: Reference group (treated with standard drug cyclophosphamide)

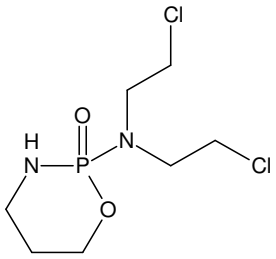
Group 3: Treated group (0.5mg/kg b.wt)

Group 4: Treated group (0.25mg/kg b.wt)

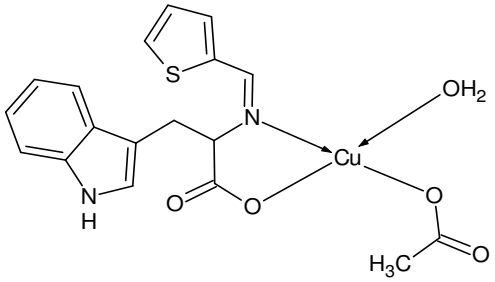
The animals were kept in well ventilated cages and provided with food and water regularly. All animals were injected with ($1x10^6$) viable Dalton's lymphoma ascites (DLA) cells in phosphate buffered saline (PBS). The tumour cells were aspirated from 15 day old DLA tumour mice. The concentration of the cells was adjusted with the help of the haemocytometer. Group 1 that received only the DLA cell line served as control. Group 2 was taken as the reference group. After 24 hours of tumour inoculation the drug was given as an injection into the intraperitoneal cavity of each animal of group 3 and group 4. Group 3 animals were injected with 100µl drug solution of 0.5mg/kg b.wt concentration and group 4 animals were injected with 0.25mg/kg b.wt drug solution. The reference group was treated with the standard drug cyclophosphamide (25mg/kg b.wt). The injections were continued for five alternate days. Then the mice were observed every day. Mean survival rate in each group was calculated by determining the standard deviations. The percentage of increase of life span (ILS) of animals treated with the standard and new drugs was calculated using the formula

$$\% \text{ ILS} = \frac{[T-C]}{C} \text{ X } 100$$

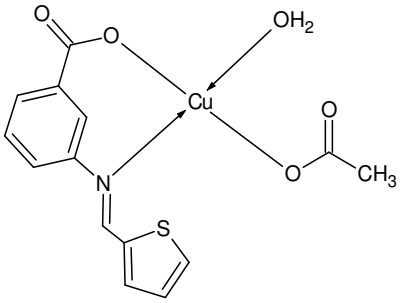
where T and C are mean survival rate of treated and control mice respectively.



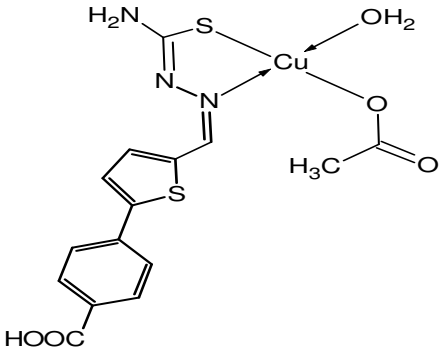
Cyclophosphamide



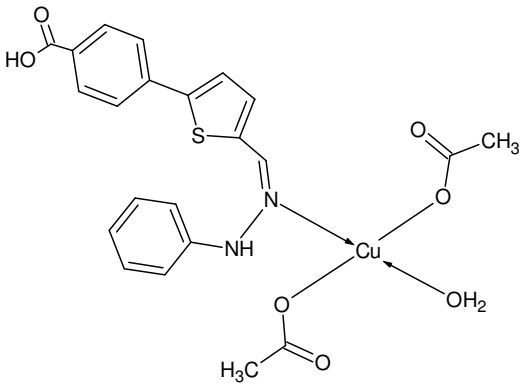
Cu(II) complex of I3YT2YMAPA



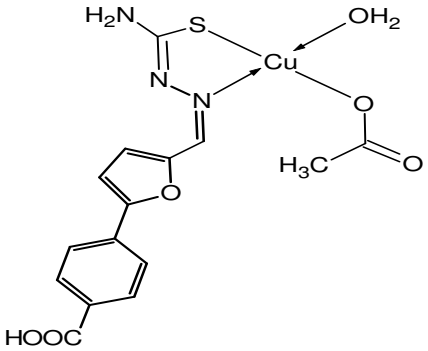
Cu(II) complex of T2YMABA



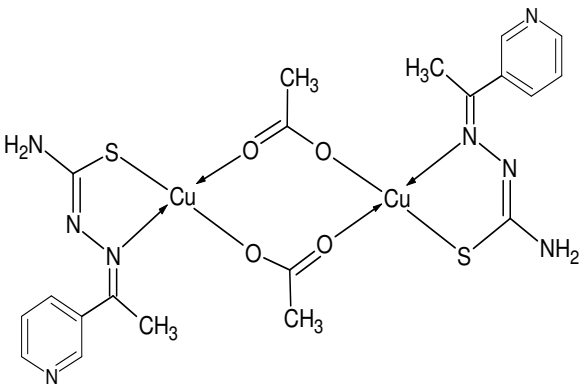
Cu(II) complex of CTHMT2YBA



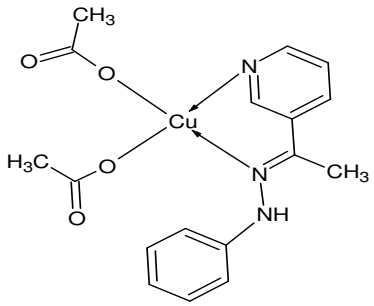
Cu(II) complex of PHMT2YBA



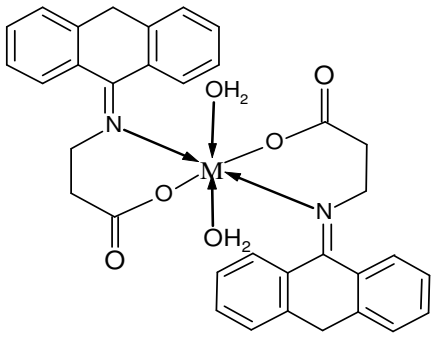
Cu(II) complex of CTHMF2YBA



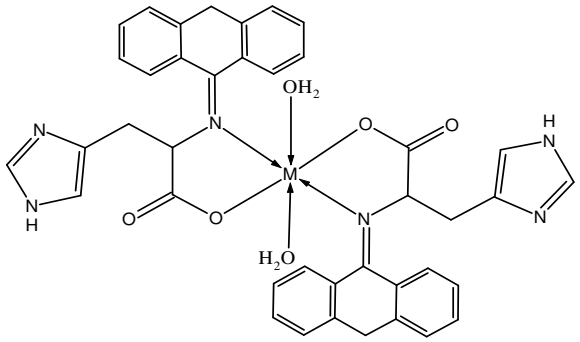
Cu(II) complex of P3YEHCTA



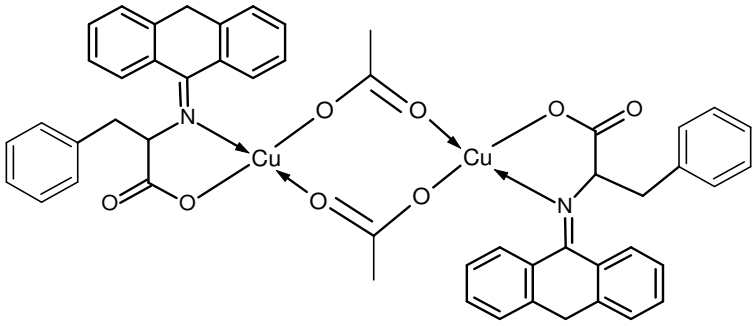
Cu(II) complex of PHEP



Cu(II) complex of A9Y3APA



Cu(II) complex of A9Y3IMPA



Cu(II) complex of A9Y3PPA

Fig. 4.2 Structures of antitumour drugs, cyclophosphamide and Cu(II) complexes of Schiff bases