Invitro Study of Ruthenium(II)-Phenanthroline-Phendione Complex on SK-MEL-28 and L6 Cell Lines

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***Abstract*-** The *invitro* antiproliferative and cytotoxic effects of $[Ru(phen)_2(phendione)]^{2+}$ (phen = 1,10-phenanthroline and phendione= 1,10-phenanthroline-5,6-dione) complex is evaluated on SK-MEL-28 melanoma and normal L6 cell lines using MTT assay and double staining method. The antiproliferative and cytotoxic effects determined using MTT assay method on both the cell lines decreases with increase in concentration of the complex. The IC_{50} value of this complex against the SK-MEL-28 cell and normal living L6 cell line is found to be 52.648 and 90.974 µg/mL respectively. Apoptosis determination using double staining method followed by fluorescent microscopic images predicts that the synthesized $[Ru(phen)_2(phendione)]^{2+}$complex shows late apoptotic effect when treated on SK-MEL-28 cells and exhibit early apoptotic effect on normal L6 cells. The apoptotic character of $[Ru(phen)_2(phendione)]^{2+}$ complex is due to the chromatin condensation of both cancerous and normal cells by the DNA binding dyes. The results revealed that the synthesized $[Ru(phen)_2(phendione)]^{2+}$ complex shows good anti-skin cancer effect on SK-MEL-28 cell line and no cytotoxicity on L6 cell line. Thus the synthesized complex can be therefore suggested as an effective anti-skin cancer drug.

***Index Terms*-** $[Ru(phen)_2(phendione)]^{2+}$complex, SK-MEL 28 cell line, L6 cell line, *Invitro*-antiproliferative effect, Cytotoxicity.

I. INTRODUCTION

Melanoma is a type of skin cancer with an increasing incidence and mortality rate [1]. The increased risk of melanoma is mainly related to UV exposure [2]. Melanoma can be cured by surgical excision at the early stage, however, later stages with distant metastasis is currently incurable. The development of agents capable of triggering cancer cell apoptosis may represent a novel therapeutic approach for melanoma treatment. An anticancer agent without affecting normal cells become effective and novel cytotoxic drug with low side effects on immune system induces apoptosis in cancer cells. Therefore, the search for new agents with a potential anti-melanoma effect is encouraged [3].

Ruthenium(II)-polypyridyl complexes are active against some cisplatin resistant cell lines and shows low side effects due to their higher selectivity for cancer cells compared with normal cells. Ruthenium can mimic iron in binding to some biological molecules [4]. Ruthenium (II) complexes with polypyridine ligands is of great interest due to their therapeutic values, DNA intercalation, protein binding and pharmacological applications. A series of octahedral ruthenium(II)-polypyridyl complexes containing N,N-chelating ligands, such as 2,2′-bipyridine (bpy), 1,10-phen anthroline (phen) etc are investigated based on their structure-activity relationships in DNA-binding properties and *invitro* cytotoxic effects toward human cancer cells**.** Therefore, Ru(II) complexes is used as an alternative to platinum complexes in cancer therapies possessing several favourable physico-chemical properties and biological applications [5,6].

Phendione a versatile bis-chelating ligand and organic linker focus mainly on the assembly of metal organic materials. The unique properties of phendione, as chelating agents plays a major role in complex chemistry. The diketone functionality can be easily transformed to other chelating groups such as a diamine or dioxime. Phendione act as redox active species due to the presence of quinonoid functionality and also act as a Lewis base due to the presence of diiminic nitrogen atoms. The *ortho*-quinone moiety of the phendione ligand may enhance the interaction with DNA *via* intercalation and hydrogen-bonding interactions [7,8].

Based on the literature survey, the present investigation focuses on the evaluation of antiproliferative and cytotoxic activities of $[Ru(phen)_2(phendione)]^{2+}$ (phen = 1,10-phenanthroline and phendione = 1,10-phenanthroline-5,6-dione) complex on SK-MEL-28 cell line and living L6 cells.

II. MATERIALS AND METHODS

A. Materials

$RuCl_3.3H_2O$, ligands (1,10-phenanthroline and 1,10-phenanthroline-5,6-dione) and ammonium hexafluorophosphate were procured from Sigma-Aldrich. SK-MEL-28 and L6cell lines was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in DMEM (Dulbecos modified Eagles medium, Himedia). HPLC grade solvents were used for the synthesis of the complex. The $[Ru(phen)_2(phendione)]^{2+}$ complex was synthesized by reacting $[Ru(phen)_2Cl_2]$ $2H_2O$ complex with phendione according to the procedure previously described [9].

B. Methods

The *invitro* antiproliferative effect on SK-MEL-28 cell line and cytotoxic effect on L6 cell line was carried out by direct microscopic observation method using Inverted phase contrast tissue culture microscope followed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Himedia, M-5655) assay method. The percentage cellular viability of the complex at various concentrations against the standard SK-MEL-28 and L6 cell lines at 570 nm was calculated by MTT assay method [10]. The Apoptotic effect of both SK-MEL-28 and L6 cell lines were determined by Acridine Orange (AO) and Ethidium Bromide (EB) Double staining method using a fluorescence microscope (Olympus CKX41 with Optika Pro5 camera).

C. Cells seeding in 96 well plate:

100 µLof trypsinised cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. Both cell lines were cultured individually in 25 cm^2 tissue culture flask with DMEM supplemented with penicillin (100 U/ml), streptomycin (100 µg/mL) and amphoteracin B (2.5 µg/mL) as antibiotic solutions and 10 % FBS (Fetal Bovine Serum), L-glutamine and sodium bicarbonate. Cultured cell lines were kept in a humidified 5% CO_2 incubator (Galaxy® 170 Eppendorf, Germany) at 37ºC.

D. Direct microscopic method

1 mg of $Ru(phen)_2(phendione)]^{2+}$ complex dissolved in 1 mL of 5% DMEM was filtered through 0.22 µM Millipore syringe filter to confirm the sterility. Sample solution of freshly prepared $[Ru(phen)_2(phendione)]^{2+}$ complex was diluted five times (6.5, 12.5, 25, 50, 100 µg) in 100 µL of 5 % DMEM and each concentration of 100 µL were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. The viability of both the cancer and normal cells were evaluated directly by placing the entire plate in an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICAPSTM HD camera) at an interval of 24 h up to 72 h and the microscopic observation were recorded.

E. MTT assay Method:

15 mg of MTT was reconstituted in 3 mL PBS and sterilized by filter sterilization. After 24 h of incubation period, the sample content in the wells were removed and 30 µL of reconstituted MTT solution was added to all test and cell control wells, the entire plate was gently shaken well and again incubated at 37ºC in a humidified 5% CO_2 incubator for 4 h. After the incubation period, the supernatant was removed and 100 µL of DMSO solution was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using micro plate reader at a wavelength of 570 nm.

The percentage cellular viability and growth inhibition of cells were calculated using the formulas:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

$$\% \text{ of growth Inhibition} = 100 - (\text{Test OD samples} / \text{Non-treated OD}) \times 100)$$

F. Determination of Apoptosis by Double Staining Method:

After treatment with IC_{50} concentrations of the test samples and 24 h incubation at CO_2 incubator, cells were washed by cold PBS and then stained with a

mixture of AO (100 µg/mL) and EB (100 µg/mL) at room temperature for 10 min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope [11].

III. RESULTS AND DISCUSSION

The structure of the synthesized $[Ru(phen)_2(phendione)]^{2+}$ complex is shown in **Fig.1**.

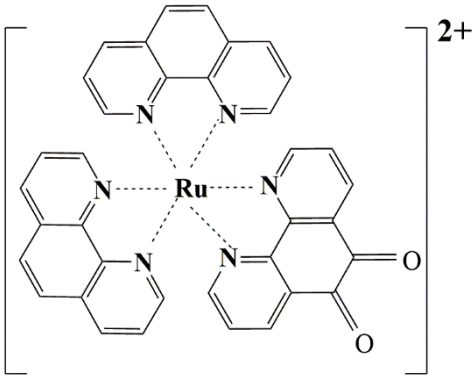

Fig.1 Structure of the$[Ru(phen)_2(phendione)]^{2+}$ complex

The absorption spectrum of $[Ru(phen)_2(phendione)]^{2+}$complexin aqueous medium shows a high energy absorption in the region 286 nm corresponding to the ligand centered π - π* transition and the low energy absorption at 440 nm assigned to the dπ - π* metal to ligand charge transfer (MLCT) transition (**Fig.2**).

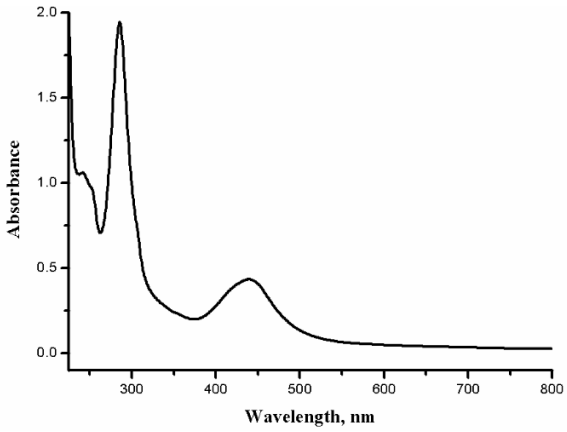

Fig.2 UV-Visible Spectrum of $[Ru(phen)_2(phendione)]^{2+}$ complex

The MLCT transition involves electronic excitation from the metal orbital [dπ (Ru)] to the ligand centered acceptor π* orbitals. The synthesized $[Ru(phen)_2(phendione)]^{2+}$complex is evaluated to examine the *invitro* antiproliferative activity on SK-MEL-28 cell line and cytotoxic activity on L6 cell line.

The morphological changes on the SK-MEL-28 cells by the addition of the complex shows cell shrinkage, blebbing followed by vacuolization (**Fig.3a-3f**).

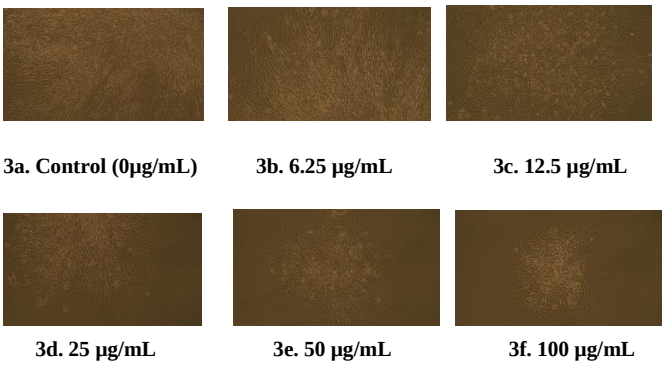

Fig.3a-3f Morphological changes on SK-MEL-28 cell line by the addition of $[Ru(phen)_2(phendione)]^{2+}$complex at various concentration

The morphological changes on L6 cells shows shrinkage initially and as the concentration of the complex increases the number of cells get reduced leading to cellular blebbing and vacuolization (**Fig.4a-4f**).

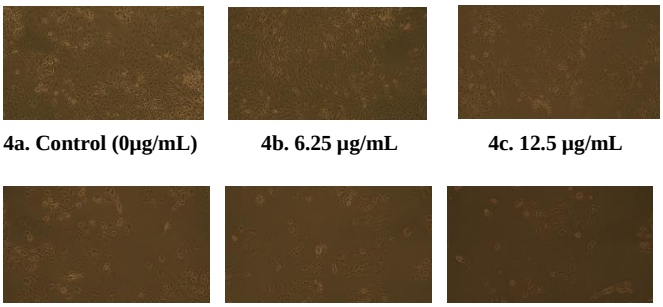

Fig.4a-4f Morphological changes on L6 cell line by the addition of $[Ru(phen)_2(phendione)]^{2+}$complex at various concentrations

This morphological changes is due to the mitochondrial depletion and ROS (Reactive Oxygen Species) production of the phendione ligand and also due to the π-π stacking interaction between the two planar phenanthroline units present in the synthesized complex. Ru(II)-polypyridyl complexes with bpy, bpz and phendione ligands on visible light excitation produce ROS. These reactive oxygen species cause extensive damage to cells and it is difficult to repair themselves by cellular machinery [12]. Hence it is reported that ROS production consequent to the cell culture treatment with the two 1,10-phenanthroline derivatives alters the metallostasis network in copper transporters and chaperones [13]. The phendione

ligand in the complex leads to cellular disruption, cell shrinkage and the formation of vacuoles which leads to cell death. Granato *et al*., have reported this type of result in phendione based compounds by disturbing the crucial physiological events of *Phialophoraverrucosa* [14]. The two carbonyl groups present in the phendione ligand does not get easily dissociate in solution instead it maintains the complex to be stable in nature and at the same time the carbonyl has the ability to interact with the ion transport channels, mitochondrial enzymes and the cellular membrane. These interactions lead to the changes in morphology of the cell lines either by disruption, cell blebbing or vacuolization [15]. Similar studies are also carried out with chalcone derivatives on A375, SK-MEL-5 and SK-MEL-28 cell lines. The chalcone derivatives damage the DNA through increased ROS levels and inhibits cell growth by inducing apoptosis [16]. After examining the morphological changes, the samples are analysed for the anti-proliferative activity on cancer cells and cytotoxicity activity on normal cells by MTT Assay method. The reconstituted and sterilized yellow MTT solution is added to the treated cell lines and untreated control cell lines individually, incubated for several hours and humidified. In active mitochondria the tetrazolium ring present in MTT gets cleaved and the reaction occurs only in living cells leads to the formation of formazan product. The formed purple colour formazan crystals incubated with live cells are then solubilized in DMSO to form a homogeneous solution and the absorbance values are measured by using microplate reader at a wavelength of 570 nm [17]. The percentage cellular viability and the percentage of growth inhibition on SK-MEL-28 and L6 cell lines are calculated from the absorbance value and is shown in **(Table 1).**

Table 1 Percentage cellular viability and percentage of growth inhibition *vs* concentration of $[Ru(phen)_2(phendione)]^{2+}$ complex on SK-MEL-28 cell line and L6 cell line

Concentration (µg /mL)	%cellular viability onSK-MEL-28 cell line	% of growth Inhibition on SKMEL-28 cell line	%cellular viability on L6 cell line	% of growth Inhibition on L6 cell line
0	100	0	100	0
6.5	91.76	8.24	93.4	6.6
12.5	81.17	18.83	82.41	17.59
25	62.35	37.65	65.93	34.07
50	42.35	57.65	47.25	52.75
100	20	80	25.27	74.73
IC_{50} (µg/mL)	**52.648**		**90.974**	

The IC_{50} value of the complex is calculated and is found to be 52.648 µg/mL for SK-MEL-28 cell line and 90.974 µg/mL for L6 cell line. The MTT assay results revealed that the percentage cellular viability decreases and the percentage growth inhibition increases with increases in concentration of the complex in both the cell lines. This may be due to the *ortho*-quinoid moiety of phendione ligand present in the $[Ru(phen)_2(phendione)]^{2+}$ complex. The obtained results confirmed that the synthesized complex shows good anti-skin cancer activity and exhibit no cytotoxicity. This implies that the $[Ru(phen)_2(phendione)]^{2+}$ complex can be used as an effective drug for the treatment of skin cancer and also melanoma related diseases.

The apoptotic behavior of the complex on both the cell lines is analysed by double staining method. The programmed cell death in both the cell lines at IC_{50} values are determined individually by fluorescent microscopy using double staining method. In the control, the living cells exhibit particularly no changes in the nuclear morphology by the AO/EB stains, but it exhibits a homogeneously stained nuclear morphology and appears as green spots [18]. After SK-MEL-28 and L6 cell lines exposed to the synthesized complex, AO is taken up by both viable and non-viable cells and emits green fluorescence by intercalating DNA whereas EB is taken up only by non-viable cells and emits orange fluorescence by fragmenting the single stranded DNA.

The fluorescent images of the synthesized complex show the inference of bright orange-stained nuclei (**Fig. 5**) with chromatin condensation indicates that the complex shows late apoptotic cell effect on cancerous SK-MEL-28 cell line.

5a. Control **5b. Test**

Fig.5 Fluorescent images of $[Ru(phen)_2(phendione)]^{2+}$ complex on SK-MEL-28 cells (a) Control (b) Test

The bright green-stained nuclei with chromatin condensation indicates early apoptotic cell effect on normal living L6 cell line (**Fig. 6**).

6a. Control 6b. Test

Fig.6 Fluorescent images of $[Ru(phen)_2(phendione)]^{2+}$ complex on L6 cells (a) Control (b) Test

Similar results are reported on SK-MEL-28 and A375 cells by chaetocin which significantly suppressed the cell proliferation and induced apoptosis in a dose and time-dependent manner [19, 20]. Thus the obtained results revealed that the synthesized $[Ru(phen)_2(phendione)]^{2+}$ complex show good antiproliferative effect and no cytotoxicity on SK-MEL-28 and normal L6 cell lines. The carbonyl groups present in the phendione ligand forms stable complex and leads to be a good model for the design of anticancer drugs. Hence the $[Ru(phen)_2(phendione)]^{2+}$ complex can be administerd as an effective anti-skin cancer drug to inhibit the growth of skin related sores and cancers.

IV. CONCLUSION

The present investigation deals about the *invitro* antiproliferative and cytotoxic effects of $[Ru(phen)2(phendione)]^{2+}$ complex on SK-MEL-28 and normal L6 cell lines. The morphological detection of the malignant SK-MEL-28 and normal L6 cells treated with various concentrations of $[Ru(phen)2(phendione)]^{2+}$ complex shows mitochondrial condensation and cell shrinkage due to the production of ROS by the phendione ligand present in the complex. The formation of formazan crystal and the absorbance of solublised formazan crystals in DMSO indicates cell inhibition and percentage viability. The IC_{50} value of the complex on SK-MEL-28 and normal L6 cells are found to be 52.648 and 90.974 µg/mL. The *invitro* anti-proliferative activity of the complex on SK-MEL-28 melanoma cells shows a bright–orange stained chromatin condensation indicates late apoptotic effect. The cytotoxic activity of the complex on L6 cells shows a bright–green stained chromatin condensation indicates early apoptotic effect. Thus, the results revealed that the synthesized complex shows good anti-skin cancer effect and no cytotoxicity towards living cells and can be suggested as an effective anti-skin cancer drug.

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