Antitumor effects of osmium (II) and ruthenium (II) bipyridine complexes containing the acetylacetonato ligand against the growth of Eherlich Ascites Cell Carcinoma

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Abstract: The development of metal-based antitumor drugs has been stimulated by the clinical success of cisplatin and its analogs and by the clinical trials of other platinum and ruthenium complexes with activity against resistant tumors and with reduced toxicity of normal cells. In the present study, the newly synthesized [OsII(bpy)2(acac)](PF6) and [RuII(bpy)2(acac)](PF6) complexes were tested for their cytotoxicities against Eherlich Ascites Cells (EAC) carcinoma, for their superoxide dismutase (SOD)- like activities and for their cytoprotective effects of the normal human red blood cells (RBCs) against photo-irradiation induced by UV-lamb in the presence of m-chloroperbenzoic acid, in vitro. Also, their killing capabilities for the growth of EAC carcinoma in vivo and the measurements of the biochemical changes accompanying such killing were investigated. The in vitro study revealed that the average cytoprotective effects of RBCs, SOD- like activities and the cytotoxicity of EAC by similar concentrations of rutheniumII (RuII) and osmium (OsII) complexes were 91.5%, 89.5% and 90% and 98.3%, 89.8% and 92.8%, respectively. In the in vivo study, the mean SOD activities in both RBCs and liver of the tumorized mice were statistically significantly inhibited compared with those of the control group ($P<0.0001$). After treatment either with RuII and OsII complexes, the activities of the latter enzyme in RBCs and liver were elevated ($P<0.0007$, $P<0.04$ and $P<0.09$ and $P>0.05$, respectively). Also, the mean activity of catalase was inhibited in liver tissues in the tumorized animals and re-elevated after complexes treatment. In addition, treatment with these complexes elevate the glutathione (GSH) levels in liver tissues of the tumorized and normal mice with simultaneous reduction in the mean levels of the corresponding values of malondialdehyde. On the other side, the mean levels of triglycerides and cholesterol were reduced in serum but the mean levels of total lipids and total proteins were elevated in liver tissues after treatment. Moreover, the mean levels of DNA and RNA were significantly elevated in liver tissues of the tumorized animals and significantly reduced after treatment of the tumorized mice with the complexes. The previous results reflect tumor growth inhibition and prevention of EAC carcinoma metastasis into the liver. In conclusion, RuII and OsII bipyridine complexes are promising free radical scavengers in phototherapy and may be used as anti-tumor and anti-metastatic agents in the clinical trials in the future.
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1. Introduction

Transition-metal-based compounds constitute a discrete class of chemotherapeutics which were widely used in the clinic as antitumor and antiviral agents. However, drug resistance and side effects have limited their clinical utility (Chen et al., 2003). These limitations have prompted a search for more effective and less toxic metal-based antitumor agents. The wide range of coordination numbers and geometries, accessible redox states, thermodynamic and kinetic characteristics, and the intrinsic properties of the cationic metal ion and ligand itself offer the medicinal chemist a wide spectrum of reactivities that can be exploited. Although metals have long been used for medicinal purposes in a more or less empirical fashion (Thompson and Orvig, 2006), the potential of metal-based anticancer agents has only been fully realized and explored since the landmark discovery of the biological activity of cisplatin (Jung and Lippard, 2007). Recently, some of the efforts have been directed to ruthenium complexes. This is because these complexes demonstrate similar ligand exchange kinetics to those of platinum (II) while displaying only low toxicity. In addition, the redox potential between the different accessible oxidation states occupied by ruthenium complexes enables the body to catalyze oxidation and reduction reactions, depending on the physiological environment (Dougan et al., 2008). Moreover, the biochemical changes that accompany cancer alter physiological environment, enabling ruthenium complexes to be selectively activated in cancer tissues (Peacock and Sadler, 2008). Also, Brabec and Nováková in 2006 found that, ruthenium compounds bind to DNA affecting its conformation differently than cisplatin and its analogues. In addition, non-nuclear targets, such as

the mitochondrion and the cell surface, have also been implicated in the antineoplastic activity of some ruthenium complexes. Brabec and Nováková (2006) added that, some chemical properties make ruthenium compounds well suited for medicinal applications and as an alternative to platinum antitumor drugs in the treatment of cancer cells resistant to cisplatin.

Superoxide radical (O_2^{-}) is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell. Therefore, SOD as a defense mechanism, is found in all subcellular locations (Alscher et al., 2002). It was showed that, a shift to a more oxidative state might result in uncontrolled lipid peroxidation, protein oxidation and ultimately cell death (Halliwell, 1999). For these reasons, the search for novel organometallic complex that defend against ROS and acting as anti-tumor agents must be the target of many studies including this study. In this study, the biological effects of osmium (II) complex with the structure [OsII(bpy)2(acac)](PF6) and ruthenium (II) complex with the structure [RuII(bpy)2(acac)](PF6) where (bpy = 2,2'-bipyridine, acac)= acetylacetone, and PF6= hexafluorophosphate) were evaluated both in vivo and in vitro. These complexes were tested in vitro for their cytotoxicities against Eherlich Ascites Cells (EAC) carcinoma, for their SOD-like activities and for their cytoprotective effects of the normal human red blood cells (RBCs) against photo-irradiation induced by UV-lamb in the presence of m-chloroperbenzoic acid. Also, their killing capabilities for the growth of EAC carcinoma and the measurements of the biochemical changes accompanying such killing were investigated in vivo.

2. Materials and methods

A-Materials:

1-Animals and tumor cell line: Adult female Swiss common bred albino mice purchased from Theodore Bilharz Institute, Giza, Egypt, with an average body weight of 25 to 30 gm were used. Ehrlich ascites carcinomas (EAC), a mammary origin, were used to give liquid tumor. These cells were kindly supplied by Doctor C. Benckhujsen, Netherland Cancer Institute, Amsterdam, Netherland. The tumor line was maintained in the Oncology Unit at the Egyptian National Cancer Institute, by serials of intraperitoneal (I.P.) transplantation in female Swiss Albino mice at 7 to 10 days intervals since 1982 up till then and was kindly supplied by such Institute. The mice were randomly divided into six groups (eight mice each) namely; normal mice (group 1), normal mice complex-treated (group 2), normal mice dimethy sulphoxide (DMSO)-treated (group 3), tumor bearing-complex treated mice (group 4), tumor-bearing-DMSO treated mice (group 5) and tumor-bearing mice only (group 6). The mice of the last 3 groups were i.p. inoculated with 1×10^6 EAC cells to produce the liquid tumor. 24 hours after tumor inoculation, the mice of group 2 and group 4 were i.p. treated with the OsII or RuII complex with a daily dose of 10 mg/kg/day (1/5 of LD50) day after day for 14 days starting from the first day after tumor inoculation. The normal- complex treated mice's group was treated with the same complex's dose as that of group 4.

2-Collection of samples: One day after the last treatment, the ascitic fluids containing EAC cells were collected and their volumes were measured. Livers were quickly dissected, rinsed with isotonic saline and dried. Then, 10 % liver tissues homogenized in cold phosphate-buffer (w/v) were prepared. After the removal of the cellular depris via centrifugation, the supernatants were used for biochemical analysis. Blood samples were also collected by tail vein cutting and their sera were used for subsequent analysis.

B-Methods:

1-Source and synthesis of the complexes:
The new complexes, [RuII bpy)2L](PF6) and [OsII (bpy)2L](PF6) (where, RuII= ruthenium(II), OsII= Osmium(II), bpy= 2,2- bipyridine, L= acetylacetone and PF6= hexafluorophosphate) have been prepared and characterized by spectroscopic measurements and also investigated by cyclic voltammetry by El-Hendawy et al. (1997) and El-Hendawy (2011), respectively. These complexes were kindly provided by Dr/ Ahmed El-Hendawy, Faculty of Science (Damietta), Mansoura University, Egypt and were used in both the in vitro and in the in vivo treatment of EAC carcinoma in the present study.

2- In vitro study:

2-1-Preparation of red blood cells (RBCs) samples: Heparinized fresh blood samples were withdrawn from five healthy volunteers and centrifuged at 3000 rpm/min. The pellets were separated and washed 3 times with phosphate buffered saline (PBS, pH 7.4, 0.01 M containing 0.135 NaCl) and centrifuged again. The cells were resuspended in PBS and 1x 106 cells were used in fluorohemolysis and the antihaemolytic effects of the RuII and OsII complexes in vitro.

2-2-Effects of m-chloro-perbenzoic acid (m-CPBA) on the fluorohemolysis and evaluation of the antihaemolytic effects of the complexes: To test

the antihaemolytic effect of the Ru(II) and Os(II) complexes, a photohaemolytic damage of normal human red blood cells (RBCs) was performed by exposing these cells to a UV-lamp in the above PBS containing 200 µM of m-CPBA (the acid concentration which gave the maximum haemolytic effect) for 30 minutes. After the completion of the incubation period, the tubes were centrifuged and the absorbance of the supernatants, as a measure of the photohaemolytic effect, was read at 546 nm in each case (Dacie and Lewis, 1984).

2-3- SOD and SOD-like activities of the complexes: SOD-like activities of the complexes and that in liver haemogenate were assayed by the method of Dechatelet et al. (1974). Simply each complex was added to a mixture of nitro blue tetrazolium salt and NADH in a pyrophosphate buffer (pH 8.3).The changes in the optical density was recorded/minute after the addition of phenazine methosulphate. The percent of inhibition of the colour development was calculated based on that of a control tube containing no complexes.

2-4-EACs cytotoxicity in vitro: The cytoxicity was determined using trypan blue exclusion by the method of MacLimans et al. (1957).

3- In vivo study:

3-1-Tumor volume: The tumor volumes were volumetrically measured in each case.

3-2-Antioxidants:

3-2-1-SOD and catalase activities in liver tissue haemogenate and SOD in RBCs: SOD activity in liver homogenate was assayed by Dechatelet et al. (1974) and that in RBCs was assayed by the procedure of Winterbourn et al. (1975). The catalase activity was determined according to Chance and Mackley (1955).

3-2-2-Glutathione (GSH) in liver tissue haemogenate and in RBCs and malondialdehyde (MDA) in liver tissues: GSH was determined in liver tissues and RBCs by the method of Beutler et al. (1963) but MDA was determined by the method of Stock and Donnandy (1971).

3-3-DNA and RNA contents of the liver tissues: The levels of DNA in liver tissues were evaluated according to Dische and Schwartezm (1937) and the RNA content was measured by the orcinol procedure of Mejbaum (1939).

3-4-Lipids profile and total proteins: The serum cholesterol was determined according to Richmond (1973) and the triglycerides were enzymatically hydrolyzed and determined according to the method of Fossati and Principe (1982). The total lipids and total proteins contents of liver tissues were determined by the methods of Knight et al. (1972) and Lowry et al. (1951), respectively.

3-5-Liver functions: Serum albumin was done according to the method of Doumas et al. (1971). Also, the activities of serum glutamic pyruvic transaminase and that of gamma-glutamyl transpeptidase (GGT) were determined by the method of Reitman and Frankel (1957) and by Szasz et al. (1969), respectively.

4-Statistical analysis: The biochemically collected data were characterized by their mean and standard deviations using instat software, version 2.03 (Graphed, USA). In addition, the student t-test was evaluated and the one-tailed P-values were also used for the statistical analysis of the results. The probability values at 0.05 up to more than 0.001 levels were considered statistically significant and 0.001 or less were considered highly significant (Snedecor and Cochran, 1969).

3. Results

1-Antihaemolytic, superoxide dismutase (SOD)-like activity and cytotoxicity effects of the complexes: The *in vitro* study revealed that the average cytoprotective effects of the complexes for the human RBCs from the photo-irradiative damage induced by UV-lamb in the presence of m-CPBA were 91.5% and 98.3 %. Also, the SOD-like activities and the cytotoxicity effects of the complexes for the viable EAC by similar concentrations of RuII and OsII complexes were 89.9% and 89.8% and 90 % and 92.8 %, respectively**.** In addition, the antihaemolytic and the cytotoxicity effects of the OsII complex were higher than those of the RuII complex **(Table 1).**

2-The activities of superoxide dismutase in red blood cells (SOD/RBCs) and the activities of both superoxide dismutase (SOD/Liver) and catalase in liver tissues: In the *in vivo* study, RuII and OsII complexes reduce the activities of SOD/RBCs, SOD/Liver and catalase in liver tissues and causes no change in the mean blood malondialdehyde (MDA) levels in the normal mice' group. On contrary, the mean activities of the former enzymes in the tumorized-non treated mice were statistically

significantly inhibited compared with those of the control group (P<0.0001). On the other hand, after treatment of the tumorized mice either with RuII and OsII complexes, the activities of these enzymes were re-elevated (P<0.0007, P<0.04 and P<0.0002 for RuII and P<0.09, P<0.13 and P<0.0005 for OsII, respectively (**tables 2 and 3**).

3-Glutathione reduced form (GSH) and malondialdehyde (MDA) in RBCs and in liver tissues: From **tables 2 and 3**, treatment with the divalent complexes elevates the mean GSH levels in liver tissues of the normal-complex treated compared to that of the normal nontreated group (P<0.0015 and P<0.0009, respectively) and in the tumorized-treated mice compared to that of the tumor nontreated group (P<0.0001 and P<0.014, respectively). In addition, simultaneous reductions in the mean MDA levels in liver tissues of the tumor-bearing mice compared to the tumorized-treated group were observed (P<0.009 and P<0.0001, respectively **and** tables 2 and 3).

4- Levels of DNA and RNA: The mean levels of DNA and RNA were significantly elevated in liver tissues of the tumorized mice compared with those of normal liver tissues (P<0.0001) and significantly reduced after complexes treatment compared with those of the tumorized non-treated mice (P<0.007 and P<0.01 for RuII and OsII complexes, respectively), a phenomenon which reflects inhibition of the tumor growth. In addition, OsII caused less damage of DNA of normal cells compared to RuII (P<0.05 and table 4 and 5).

5- Levels of lipids profile and the total proteins in liver tissues: Firstly, the treatments of normal mice with any of the complexes (OsII and RuII complexes, respectively) significantly reduce their mean serum levels of triglycerides (P<0.01 and P<0.33) and total lipids in their liver tissues (P<0.05 and P<0.03) compared with those of the normal control group. In addition, after killing of the tumor cells by the complexes, the mean serum levels of triglycerides (P<0.0001 in each case) and cholesterol in their sera were significantly reduced (P<0.04 in case of RuII only) but the mean levels of total lipids (P<0.08 and P<0.002, respectively) and total proteins (P<0.0001 in each case) were elevated in the same organ compared with those of the tumorized non-treated mice (**Table 4 and 5)**.

6- Liver function tests:

6-1- Serum albumin: The tumorized- non-treated mice showed significantly lowered albumin levels in their sera compared with that of normal mice (P<0.005), indicating a state of liver damage due to metastasis of EAC into such organ. In addition, complexes treatment did not affect serum albumin levels **(P<0.4** and **tables 6 and 7**).

6-2- Serum γ-GT and serum glutamic pyruvic transaminase (SGPT**):** As shown in tables 6 and 7, γ-GT mean activities are much elevated in sera of the tumorized non-treated mice than that of the normal controls (P<0.0001). In addition, the treatment with the complexes caused dramatic decrease in γ-GT activities compared with that of the tumorized non-treated mice (P<0.0001). On the other hand, DMSO did not affect the latter enzymes activities in the normal-DMSO treated animals. On the other side, the normal mice treated with the complexes showed reduction in the mean activities of catalase (P<0.01 in case of RuII complex) and slight elevations in the mean activities of SGPT compared with those of the normal control. Surprisingly, OsII complex is less toxic to normal liver cell compared with RuII one. This is because the mean activity of SGPT in normal OsII- treated mice is lowered than that of normal RuII- treated mice (P< 0.053 and **tables 6 and 7**).

6- Tumor volume: The mean volumes of the ascitic fluids after treatment of any of the complexes were highly significantly reduced (P<0.0001) compared with those of the tumorized non-treated mice **(Tables 6 and 7**).

4. Discussions

The field of medicinal inorganic chemistry is rapidly advancing. In particular organometallic complexes have much potential as therapeutic and diagnostic agents (Peacock and Sadler, 2008). The development of metal-based antitumor drugs has been stimulated by the clinical success of cisplatin in the treatment of resistant tumors and by the clinical trials of other platinum and ruthenium complexes showing reduced toxicities Brabec (2002). It is therefore of great interest, in this study, to understand the details of molecular and biochemical mechanisms underlying the biological efficacy of ruthenium and osmium complexes both *in vitro and in vivo*.

In the present study, two complexes of RuII and OsII with the same ligand were tested in vitro for their capabilities to prevent the photohaemolysis of human RBCs sensitized by m-CPBA via scavenging the produced free radicals, to mimic SOD activity and to kill EAC carcinoma. In this study, it was found that the complexes scavenge the free radicals produced from the photosensitization of m-CPBA

Table 1: Antihaemolytic effects, superoxide dismutase (SOD)-like activities and cytotoxicity of ruthenium and osmium bipyridine complexes.

Effects / Volume	Antihaemolytic effects©		SOD-like activities©		Cytotoxicity©	
	RuII	OsII	RuII	OsII	RuII	OsII
20 µL	90.3 %	97.7 %	78.2 %	76.9%	82 %	86 %
50 µL	91.3 %	98.1%	87.1 %	79.5 %	89 %	92 %
100µL	91.8 %	98.4 %	91.2%	96.2 %	91%	94 %
150 µL	91.9 %	98.6 %	93 %	96.6 %	94 %	96 %
200 µL	92.0 %	98.6 %	100%	100%	94%	96 %
Average effects %	91.5 ± 0.7	98.3 ± 0.4	89.9 ± 8.0	89.8 ± 10.8	90 ± 4.9	92.8 ± 4.1
Effects' range %	91.3 - 92	97.7 - 98.6	78.2 - 100	76.9 - 100	82 – 94	86 – 96

© = Values are the average of 5 different readings.

Table (2): Mean activities of superoxide dismutase in both red blood cells (SOD/RBCS) and liver tissues (SOD/Liver), catalase in liver tissues and the mean levels of both glutathione reduced form (GSH) and malondialdehyde in RBCs and in liver tissues of mice treated with Ruthenium bipyridine complex.

Parameters / *Group*	SOD/RBCS (U/0.01 gm haemoglobin)	SOD/Liver (% of inhibition/ 0.01 gm tissue)	Catalase (U/0.01 gm tissue)	GSH /RBCS (Mol/L packed cells)	GSH /Liver (mmol/ gm protein)	MDA/RBCs (Mol/ml packed cells $\times 10^{-5}$)
Normal	8.7 ± 1.8 (7.0 -10)	58 ±11 (43 - 70)	21 ± 5.0 (13 - 25)	0.8 ± 0.1 (0.6 - 0.8)	2.3 ± 0.4 (1.7- 2.7)	0.8 ± 0.2 (0.7 - 1.1)
Normal + DMSO	7.5 ± 2.6 (4.0 -10)	47 ± 8.0[i] (39 - 54)	20 ± 4.0 (15 - 24)	0.9 ± 0.2 (0.7 - 1.0)	2.2 ± 0.5 (1.6 - 2.7)	0.8 ± 0.3 (0.5 - 1.2)
Normal + Ru(II) treatment	6.3 ± 2.5[i] (4.0 - 10)	40 ± 8.0[i] (34 - 53)	14 ± 3.0[i] (10 - 17)	0.6 ± 0.1[i] (0.4 - 0.8)	3.9 ± 1.2[i] (2.7 -5.7)	1.0 ± 0.1[i] (0.9 - 1.1)
Tumor only	3.7 ± 0.8[ii] (2.9 - 5.0)	27 ± 7.0[ii] (21 - 37)	7.0 ± 4.0[ii] (5.0 - 13)	0.6 ± 0.1[i] (0.5 - 0.7)	1.5 ± 0.8[i] (0.5 - 2.4)	2.0 ± 0.2[ii] (1.7 - 2.2)
Tumor + DMSO	3.6 ± 1.8[ii] (2.0 - 7.0)	28 ± 7.0[ii] (21 - 37)	7.0 ± 3.0[ii] (5.0 - 11)	0.7 ± 0.2 (0.5 - 1.0)	1.2 ± 0.6[ii] (0.6 - 1.9)	2.0 ± 0.3[ii] (1.6 - 2.3)
Tumor + Ru(II) treatment	7.5 ± 2.6[i,**] (4.0 -10)	36 ± 11[ii,*] (19 - 46)	16 ±4.0[i,**] (12 - 23)	0. 5 ± 0.2[ii] (0.3 - 0.6)	3.5 ± 0.6[ii,**] (3.1- 4.5)	1.4 ± 0.6[i,*] (0.4 - 1.8)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (3): Mean activities of superoxide dismutase in both red blood cells (SOD/RBCS) and liver tissues (SOD/Liver), Catalase in liver tissues and the mean levels of both glutathione reduced form (GSH) and malondialdehyde in RBCs and in liver tissues of mice treated with osmium bipyridine complex.

Parameters / *Group*	SOD/RBCS (U/0.01 gm haemoglobin)	SOD/Liver (% of inhibition/ 0.01 gm tissue)	Catalase (U/0.01 gm tissue)	GSH /RBCS (Mol/L packed cells)	GSH /Liver (mmol/ gm protein)	MDA/RBCs (Mol/ml packed cells $\times 10^{-5}$)
Normal	8.7 ± 1.8 (7.0 -10)	58 ±11 (43 - 70)	21 ± 5.0 (13 - 25)	0.8 ± 0.1 (0.6 - 0.8)	2.3 ± 0.4 (1.7- 2.7)	0.8 ± 0.2 (0.7 - 1.1)
Normal + DMSO	7.5 ± 2.6 (4.0 -10)	47 ± 8.0[i] (39 - 54)	20 ± 4.0 (15 - 24)	0.9 ± 0.2 (0.7 - 1.0)	2.2 ± 0.5 (1.6 - 2.7)	0.8 ± 0.3 (0.5 - 1.2)
Normal + Os(II) treatment	5.6 ± 2.8[i] (3.3 - 10.0)	31 ± 9.4 (21 - 43)	± 3.0 16 (10 - 19)	0.8 ± 0.2 (0.6 -1.1)	3.5 ± 0.8[ii] (2.7 - 4.5)	1.0 ± 0.1[i] (0.9 - 1.1)
Tumor only	3.7 ± 0.8[ii] (2.9 - 5.0)	27 ± 7.0[ii] (21 - 37)	7.0 ± 4.0[ii] (5.0 - 13)	0.6 ± 0.1[i] (0.5 - 0.7)	1.5 ± 0.8[i] (0.5 - 2.4)	2.0 ± 0.2[ii] (1.7 - 2.2)
Tumor + DMSO	3.6 ± 1.8[ii] (2.0 - 7.0)	28 ± 7.0[ii] (21 - 37)	7.0 ± 3.0[ii] (5.0 - 11)	0.7 ± 0.2 (0.5 - 1.0)	1.2 ± 0.6[ii] (0.6 - 1.9)	2.0 ± 0.3[ii] (1.6 - 2.3)
Tumor + Os(II) treatment	4.7 ±1.8[ii] (3.3 – 6.7)	31 ± 7.0[ii] (22-38)	13 ± 4.0[i,*] (8.0 - 19)	0.6 ± 0.1[ii] (0.6 – 0.7)	2.2 ± 0.1[*] (2.0 - 2.4)	1.4 ± 0.1[ii,**] (1.2 - 1.6)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (4): Mean serum levels of triglycerides and cholesterol and the mean total Lipids, DNA and RNA in liver tissues of mice treated with ruthenium bipyridine complex.

Parameters / Group	*Tri- glycerides* (mg %)	*Cholesterol* (mg %)	*Total Lipids* (mg / gm tissue)	*Total proteins* (mgm/gm tissue)	*DNA* (mgm/gm tissue)	*RNA* (mgm/gm tissue)
Normal	118 ± 17 (101-142)	130 ± 35 (96-185)	18 ± 5.0 (13 - 26)	388 ± 5.0 (382 -395)	9.0 ± 2.0 (7 - 11)	27 ± 5.0 (21 - 32)
Normal + DMSO	105 ± 16 (80-121)	158 ± 32 (111-190)	20 ± 4.0 (13 - 24)	380 ± 4.0[i] (375-386)	9.0 ± 2.0 (7 - 18)	28 ± 4.0 (24 - 32)
Normal + Ru(II) treatment	124 ± 34 (99-175)	123 ± 27[i] (95-166)	14 ± 2.0[i] (11 - 18)	382± 2.0[i] (380-384)	14 ± 4.0[i] (8 - 18)	29 ± 3.0 (28 - 34)
Tumor only	216 ± 40[ii] (180-260)	126 ± 29 (99-167)	11 ± 5.0[i] (6 -16)	368 ± 5.0[ii] (360-372)	19 ± 4.0[ii] (15 - 23)	36 ± 3.0[ii] (32 - 40)
Tumor + DMSO	218 ±35[ii] (182-260)	129 ±31 (99-168)	11 ± 3.0[i] (7.0 -16)	366 ± 6.0[ii] (356- 372)	14 ± 2.0[ii] (12 -18)	36 ± 6.0[i] (26 - 40)
Tumor + Ru(II) treatment	106±26[**] (73-140)	105 ±11[*] (93-120)	19 ± 5.0[*] (15 - 27)	388 ± 2.0[ii,**] (377-382)	13± 5.0[i,*] (8 - 18)	31 ± 5.0[*] (28 - 39)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (5): Mean serum levels of triglycerides and cholesterol and the mean total Lipids, DNA and RNA in liver tissues of mice treated with osmium bipyridine complex.

Parameters / Group	*Tri- glycerides* (mg %)	*Cholesterol* (mg %)	*Total Lipids* (mg / gm tissue)	*Total proteins* (mgm/gm tissue)	*DNA* (mgm/gm tissue)	*RNA* (mgm/gm tissue)
Normal	118 ± 17 (101-142)	130 ± 35 (96-185)	18 ± 5.0 (13 - 26)	388 ± 5.0 (382 -395)	9.0 ± 2.0 (7 - 11)	27 ± 5.0 (21 - 32)
Normal + DMSO	105 ± 16 (80-121)	158 ± 32 (111-190)	20 ± 4.0 (13 - 24)	380 ± 4.0[i] (375-386)	9.0 ± 2.0 (7 - 18)	28 ± 4.0 (24 - 32)
Normal + Os(II) treatment	101 ±10[i] (88-116)	116 ± 33 (75-155)	15 ± 2.0[i] (13 - 17)	381 ± 3.0 (380 - 384)	10 ± 3.0[i] (5 - 13)	24 ± 3.0[i] (2.1-2.7)
Tumor only	216 ± 40[i] (180-260)	126 ± 29 (99 - 167)	11 ± 5.0[i] (6 - 16)	368 ± 5.0[ii] (360-372)	19 ± 4.0[ii] (15 - 23)	36 ± 3.0[ii] (32 - 40)
Tumor + DMSO	218 ±35 (182-260)	129 ±31 (99 - 168)	11 ± 3.0[i] (7.0 -16)	366 ± 6.0[ii] (356- 372)	14 ± 2.0[ii] (12-18)	36 ± 6.0[i] (26 - 40)
Tumor+ Os(II) treatment	97 ±12[i,**] (84-114)	114 ± 45 (60 - 162)	14 ± 3.0[i] (9.0 - 16)	382 ± 2.0[i,**] (377-382)	13 ± 5.0 [i,*] (9.0 -20)	29 ± 4.0[*] (26 - 35)

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (6): Mean levels of albumin and the activities of γ- glutamyl transpeptidase (γ- GT) and glutamic pyruvic transaminase in sera of mice treated with Ruthenium bipyridine complex.

Parameters / Group	Albumin *(gm%)*	GGT *(IU/l)*	SGPT *(IU/ml)*	Tumor volume *(ml)*
Normal	3.9 ± 0.3 (3.6 - 4.2)	26 ± 12 (19 - 47)	26 ± 6.0 (20-32)	--
Normal + DMSO	3.3 ± 0.6[i] (2.6 - 4.2)	27 ± 8.0 (19 - 40)	25 ± 7.0 (19-34)	--
Normal + Ru(II) treatment	3.2 ± 0.7[i] (2.1 - 3.7)	14 ± 6.0[i] (8 - 23)	35 ± 8.0[i] (22-41)	--
Tumor only	3.1 ± 0.7[i] (2.4 - 3.7)	119 ± 18[ii] (90 - 141)	37 ± 9.0[i] (26-50)	3.8 ±1.3 (2.0- 5.0)
Tumor + DMSO	2.9 ± 0.5[ii] (2.2 - 3.5)	113 ± 17[ii] (94 - 132)	41 ± 9.4[ii] (27 - 53)	3.6 ±1.14 (2.0- 5.0)
Tumor + Ru(II) treatment	3.1 ± 0.6[i] (2.4 - 3.9)	20 ± 11[**] (10 - 34)	32 ± 7.0[i] (22-41)	0.4 ± 0.22 (0.0 - 0.5)[**]

[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

Table (7): Mean levels of albumin and the activities of γ- glutamyl transpeptidase (GGT) and glutamic pyruvic transaminase in sera of mice treated with osmium bipyridine complex.

Parameters / Group	Albumin (gm %)	GGT (IU/l)	SGPT (IU/ml)	Tumor volume (ml)
Normal	3.9 ± 0.3 (3.6 - 4.2)	26 ± 12 (19 - 47)	26 ± 6.0 (20-32)	--
Normal +DMSO	3.3 ± 0.6[i] (2.6 - 4.2)	27 ± 8.0 (19-40)	25 ± 7.0 (19-34)	--
Normal + Os(II) treatment	3.36 ± 0.6[i] (2.4-3.9)	21 ± 4.0 (15-24)	27 ±9.6 (19-42)	--
Tumor only	3.1 ± 0.7[i] (2.4-3.7)	119 ±18[ii] (90-141)	37.4±9[i] (26-50)	3.8 ±1.3 (2.0- 5.0)
Tumor + DMSO	2.9 ± 0.5[ii] (2.2-3.5)	113 ±17[ii] (94-132)	40.8±9.4[ii] (27-53)	3.6 ±1.14 (2.0- 5.0)
Tumor + Os(II) treatment	3.1±0.6[i] (2.5-3.6)	28 ±11[**] (18-41)	17±5.7[i,**] (12-24)	0.6 ± 0.32[**] (0.4- 0.9)

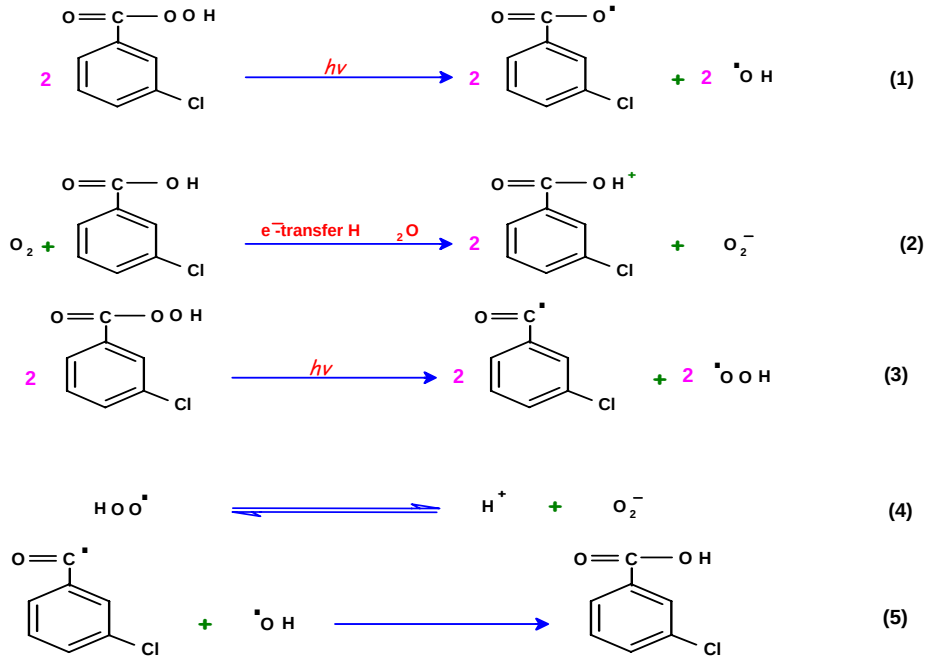
[i]= Significant and [ii]= highly significant compared with those of the control and [*]= Significant and [**]= highly significant compared with those of tumor only.

and thus protect the RBCs from subsequent haemolysis.This is because the previous evidence indicated that ROS causes photohaemolysis of human RBCs photosensitized by m-CPBA (El-Naggar, 1997 and Abou-Seif and Elgendy, 1998). Such hemolytic state may be due to the formation of direct cellular membranes injury by the formed lipid peroxides. The latter peroxide formation may be attributed to the increase in the oxidative flux (Kurata et al., 1993) produced by the excessive free radicals e.g. $O_2^{-\cdot}$, $^{\cdot}OH$ and H_2O_2 synthesized during the photohaemolytic process. In addition, the involvement of the peroxidation process in the depletion of glutathione, NADH and adenosine triphosphate (ATP) causes more cellular destruction of RBCs (Halliwell, 1999). In the present study, the mean levels of GSH were significantly elevated in normal mice treated with RuII and OsII complexes compared with those of normal mice without treatment ($P<0.0001$) indicating that these complexes can be implicated in the redox cycle involved in GSH production (Dougan et al, 2008). Another proposed mechanism of RBCs lyses may be via the hydrophobic interactions between the formed hydroperoxides produced as a result of oxidative stress on the membrane double layers of RBCs. The latter interaction facilitates the penetration of water molecules into the inside of RBCs causing more haemolytic damage (Berround et al., 1996). Abou-Seif (2004) suggested that, the main initial radical generated from fluoroirradiation of m-CPBA is the $O_2^{-\cdot}$. He also, proposed two mechanisms to explain the production of the $O_2^{-\cdot}$ and/or $^{\cdot}OH$ radicals during the fluoroirradiation. The first involves fluorogeneration of hydroxyl radical ($^{\cdot}OH$) then $O_2^{-\cdot}$ radical. The $^{\cdot}OH$ could be produced by transforming e^- from the aqueous medium to the oxygen molecule (Packer, 1993) as presented in equations 1 and 2. In the second mechanism, $O_2^{-\cdot}$ radicals are generated firstly followed by $^{\cdot}OH$ radicals through hydrogen abstraction from the medium (equations 3 and 4).

In the present study, the RuII and OsII complexes showed similar percentages of SOD- like activities of about 90%. Such results confirm the tendency of such complexes to consume $O_2^{-\cdot}$ produced during photosensitization of RBCs. The abilities of such complexes to prevent RBCs destruction by 91.5% and 98.3%, respectively also confirm the abilities of these complexes to act as free radicals scavengers and to be used in photodynamic therapy. Also, the results favor the first proposed mechanism by Abou-Seif (2004) in which the main and the initial radical generated from fluoroirradiation of m-CPBA is the $O_2^{-\cdot}$. In addition, the ability of the OsII to protect the human RBCs and also its cytotoxicity for the normal EAC was higher than those of the RuII complex. This may be due to the fact that the liability of acetylacetone ligand in OsII complex is lower than that in ruthenium analogue which is similar to that of cis-platinum compared to cis-palladium complex (Hacker et al., 1984). The latter outhers stated that, the development of pd(II) anticancer drugs has not been promising, this is because pd(II) complex are about 105 times more reactive than their Pt(II) analogues leading to rapid hydrolysis of the leaving group(s).

Kostrhunova et al. (2008) showed that OsII arene complexes bind and distort polymeric DNA with a rate of binding comparable to that of cisplatin. They added that, the extent of the interaction of these complexes with DNA were correlated well with their cytotoxicities. In addition, the latter outhers reported that the osmiated-DNA inhibits RNA synthesis like that of cisplatin and the ruthenium analogue. Moreover, these outhers added that the unwinding angle induced in supercoiled plasmid DNA by osmium (II) arene complexes is larger (21-27°) in comparison to the ruthenium analogues (7-14°) or cisplatin. Such outhers attributed this to the intercalation of the arene interacts into the duplex. Finally, they reported that complexes with extended electron-rich π-systems can displace the intercalator ethidium bromide from DNA, *in vitro*, so supporting the previous intercalation hypothesis for the previous metal complexes. In fact, the osmium (II) and ruthenium (II) bipyridine complexes of the present study contain both the arene system bipyridyl and the electron-rich π-systems; namely, acetylacetone ligand suggesting the possible involvement of the previous mechanism in EAC carcinoma killing.

Vock et al. (2006) tested ten of ruthenium arene complexes with the general formula [Ru(eta6-arene)Cl_2(L)], (arene=benzene, p-cymene; L= imidazole, benzimidazole, N-methylimidazole, N-butylimidazole, N-vinylimidazole, N-benzoylimidazole; X = Cl, BF4, BPh4) for their selectivity toward cancer cells in vitro, those which showed higher cytotoxicity to the tumor cells but they were less (or not) cytotoxic toward nontumorigenic cells have been selected for a more detailed *in vivo* evaluation. In the present study, both of RuII and OsII showed higher EAC carcinoma toxicities *in vitro*, and therefore, were more evaluated *in vivo*. The *in vivo* results of the present study showed that, the mean levels of total lipids and total proteins in liver tissues of the tumorized mice were reduced and those of triglycerides in their sera were elevated confirming the existence of a catabolic state accompanying the growth of the tumor cells (Korekane et al., 2003).



These findings were confirmed by the re-elevation of the mean levels of the formers and the reduction of the latter after tumor killing by any of the two complexes and also by the reduction of the liver DNA and RNA contents after treatment of EAC with the complexes than those of the tumorized untreated animal. The latter results led one to confirm the abilities of the studied complexes not only to treat the tumor cells but also to prevent their metastasis to the liver (Tables 4 and 5). The reduction of the mean activities of both GGT (a tumor marker and a liver function enzyme) and SGPT (a liver function enzyme) after tumor treatment with both RuII and OsII complexes compared with those of the tumorized non-treated mice can confirm the latter two abilities. In addition, γ-GT is considered to be much more sensitive than either SGPT and/ or albumin in reflecting liver affection due to EAC implantation. This is because the former enzyme, activities were much elevated in sera of the tumorized non-treated mice than that of the normal controls ($P<0.0001$). In addition, the treatment with the complexes caused dramatic decrease in GGT activities compared with the tumorized non-treated mice ($P<0.0001$ for both RuII and OsII complexes (Tables 6 and 7).

In the present study, the decrease in the mean activities of SOD in RBCs and liver and that of catalase in liver tissues of the tumorized animals can cause a state of oxidative stress and thence formation of lipid peroxide causing cellular and organ damage. Such damage may include the EAC carcinoma and liver tissues. This is actually the case, because MDA was elevated in liver tissues of the tumorized animals and the activities of both SOD and catalase enzymes were re-elevated after treatment of the tumorized mice with the complexes. Such elevations can protect liver tissues from the oxidative stress, via scavenging the substrates of the latter two enzymes; namely, superoxide radicals and the formed H_2O_2, respectively. Also, such scavenging effect may participates in EAC killing throughout the prevention of their metastasis into the liver. Alessio et al. (2004) added that the use of a well known RuII complex that successfully completed a Phase I trial had the capacity to modify important parameters of metastasis such as tumor invasion, matrix metalloproteinases activity and cell cycle progression. One cannot neglect the involvement of such mechanisms during killing of EAC by the bipyridyl acetylacetenato RuII and OsII complexes.

Moreover, Kostrhunova et al. (2008) investigated the interactions between the potential biological target DNA and four OsII arene complexes, where arene = biphenyl or p-cymene and showed that these complexes bind to DNA. In their study, some of the OsII complexes exhibit promising cytotoxic effects in ovarian tumor cell lines. They also showed that such complexes produced DNA adducts and largely distort DNA conformation. The authors concluded that, the cytotoxicities of the complexes are consistent with their DNA binding and the binding involves combined coordination to guanine residues together with noncovalent interactions between the arene ligand and the DNA. In the present study, both RuII and OsII bipyridyl complexes containing the O,O-donor; acetylacetonato ligand showed higher percentages of EAC toxicities both in vitro and in vivo. Also, Pizarro and Sadler (2009) suggested that, DNA is believed to be the primary target for many metal-based drugs. These drugs can form specific lesions on DNA that induce its apoptosis. Therefore, it was concluded that the present complexes can follow a similar mechanism to that of Kostrhunova et al. (2008) and Pizarro and Sadler (2009) during killing of EAC. This conclusion is based on the reduction in the mean levels of nucleic acids after treatment of the tumorized mice with any of the complexes compared to the non-treated mice.

Pizarro and Sadler (2009) also added that, the newly emerging ruthenium (II) complexes not only bind to DNA coordinately, but also by both H-bonding and hydrophobic interactions triggered by the introduction of the extended arene rings into their versatile structures. In the present study, the bipyridyl moities of the studied complexes can participate, at least in part, in the hydrophobic interaction of both complexes with DNA causing its damage. Intriguingly, Pizarro and Sadler (2009) and Kostrhunova et al. (2008) added that osmium (the heavier congener of ruthenium) reacts differently with DNA but can also give rise to the high cytotoxic effects of the organometallic complexes. This is already the case in the present study, because OsII complex had significantly higher superoxide scavenging activity which protect the human RBCs from the photohaemolytic effects of m-CPBA. Süss-Fink (2010) also concluded that, the neutral or cationic arene ruthenium complexes provide both hydrophilic as well as hydrophobic properties due to the robustness of the ruthenium-arene unit that hold a high potential for the development of metal-based anticancer activity against a variety of cancer cells. *In conclusion,* RuII and OsII complexes can be used as promising free radical scavengers in phototherapy and may be used as anti-tumor, with slight normal cells toxicities, and anti-metastatic agents in the clinical trials in the future.

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