Synthesis, Characterization and Antitumor Activities of 1,1`-Diacetylferrocene Dihydrazone Containing a Salicylaldehyde Moiety and its Complexes with Pd(II) and Pt(II)

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(Received on 19th December 2012, accepted in revised form 5th April 2013)

Summary A ferrocenyl ligand was prepared from condensation of 1,1`-diacetylferrocene dihydrazone with salicylaldehyde. Ligand forms 1:1 complexes with Pd(II) and Pt(II) in good yield. Characterization of the ligand and complexes was carried out using elemental analysis, infrared, ^{1}H nuclear magnetic resonance and electronic absorption spectra. Anticancer activity of the prepared ligand and its complexes against human breast cancer cell line MCF-7 was determined, and the results were compared with the activity of the commonly used anticancer drug cisplatin. The results suggested that the prepared compounds possess significant antitumor activity comparable to the activity of cisplatin and may be potent anticancer agents for inclusion in modern clinical trials.

Keywords: Bioinorganic chemistry, *Schiff* base Ligands, Metallocenes, Pd(II) and Pt(II) complexes, biometals.

Introduction

Use of ferrocene in bioorganometallic chemistry has been growing rapidly in recent years. This may be because ferrocenyl derivatives are stable compounds, nontoxic, and have good redox properties. Many ferrocenyl compounds display interesting antitumor [1-5], antimalarial [6-8], antifungal [9-10], and DNA-cleaving activity [11-13]. Along this line, Vessieres *et al.* [14] prepared several derivatives of ferrocifen based on the structure of tamoxifen and hydroxytamoxifen. The series of ferrocifens were biologically examined in vitro and in vivo, and the results [15-16] showed that ferrocifens are active against both hormone-dependent and hormone-independent breast cancer cells. Top *et al.* [16] investigated also several derivatives of polyphenolic compounds containing a ferrocene moiety and evaluated them as anticancer agents using standard breast cancer cell lines.

Another successful example is a ferrocene-chloroquine analogue, i.e., ferrochloroquine (FQ,7-chloro-4-[2-(N0,N0-dimethylaminomethyl)-N-ferrocenylmethylamino]quinoline), in which one ferrocene unit was integrated into chloroquine (CQ). In vitro, FQ proved to be about 22 times more biologically active than CQ against chloroquine-resistant strains of *P. falciparum* and showed higher activity in vivo in mice infected with *P. berghei N and P. yoelii NS* [3, 17, 18].

In addition, the redox properties of ferrocene have been exploited to prepare different types of electrochemical sensors such as DNA, protein, environmental pollutant, and microscopic biological sensor, and this area of research is growing rapidly [19-20].

These interesting applications of ferrocenyl compounds have attracted the attention of many authors to study heterobimetallic complexes [21-23], since some ferrocenyl complexes showed higher biological activity than the parent ligand.

The aim of this work is to prepare and spectral study a ferrocenyl ligand derived from condensation of 1,1`-diacetylferrocene dihydrazone with salicylaldehyde. The ligand has been well-characterized using different spectroscopic techniques. The study was extended to prepare and spectral study Pd(II) and Pt(II) complexes with the mentioned ligand.

Ligand and its complexes have been characterized by IR, ^{1}H NMR, UV-Vis spectra as well as elemental analysis. On the other hand, platinum compounds such as cisplatin, carboplatin, and oxaliplatin are some of the most potent anticancer drugs available today. These compounds are used for treatment of different cancer types, although they have several severe side effects [24]. Preparing new compounds with improved potency or wide specificity is among the major targets for pharmaceutical companies. Therefore, our aim was planned to study the antitumor activity of the ligand and complexes, comparing their activity with that of cisplatin.

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Results and Discussion

Synthesis and Characterization of Ligand

1,1`-Diacetylferrocene dihydrazone was prepared by reacting 1,1`-diacetyl-ferrocene, dissolved in a small amount of dry ethanol, with an excess of hydrazine hydrate while stirring under nitrogen. The deep-brown color of the diacetylferrocene started to change to orange within the first 4-5 h, but stirring was continued to complete the reaction [5]. Characterization of ferrocenyl dihydrazone was carried out using IR, ^{1}H NMR, and UV-Vis spectra. The IR spectra of the prepared dihydrazone showed a medium band at 1,663 cm^{-1}, which was assigned to the formation of the C=N group [22-23]. In addition, the bands of the two NH_2 groups appeared as a broad signal at about 3,331 and 3,199 cm^{-1} [25]. This result was confirmed by the broad ^{1}H NMR band at 5.08 ppm, which was assigned to the NH_2 groups of the 1,1`-diacetylferrocene dihydrazone.

The ligand (Fig. 1) was prepared by addition of salicylaldehyde to 1,1`-diacetylferrocene dihydrazone in ethanol under reflux for 8 h. The color of the dihydrazone was changed to orange-red. The ligand was isolated in good yield and is soluble in common organic solvents. Structure of the ligand was confirmed by IR spectra. The band at 1,615 cm^{-1} due to the C=N stretching vibration was found to increase in intensity [22]. This may be due to the formation of another two C=N bonds in the ligand. A broad band centered at 3,425 cm^{-1} indicated the presence of the OH group in the ligand. The breadth of this band also suggested the presence of hydrogen bonding between the azomethine nitrogen and the OH group [26]. A broad band centered at 1,042 cm^{-1} was observed with medium intensity and was assigned to the N-N group [27]. In the ^{1}H NMR, the protons of the cyclopentadienyl groups appeared at 4.65 and 4.39 ppm. The phenyl protons were noticed as new bands at 7.56-6.82 ppm. The proton in the H-C=N group appeared as a singlet at 8.61 ppm, whereas the phenolic OH appeared at 9.72 ppm in the ^{1}H NMR. This peak was confirmed from other ^{1}H NMR spectra of similar *Schiff* bases [21, 22].

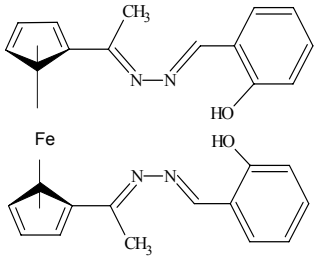

Fig. 1: Structure of the ligand.

Synthesis and Characterization of the Complexes

The complexes of palladium (II) and platinum (II) were prepared in good yield from an equimolar ratio of the ligand and the corresponding metal (II) chloride as discussed in the experimental part. The two complexes are very deep red, which may be due to conjugation of the ligand with the metal ions. The complexes are also stable in air and under light, and are soluble in dimethylformamide (DMF) and dimethyl sulfoxide (DMSO). Elemental analysis showed that the complexes have 1:1 (metal:ligand) molar ratio.

The IR spectra of the complexes were recorded as KBr pellets. It was found that the characteristic band of the C=N group in the free ligand (at 1,615 cm^{-1}) was shifted to lower frequency of 1,602-1,606 cm^{-1} in the complexes [22]. This shift indicates coordination of the azomethine nitrogen to the metals in the complexes. It was also found that the medium band due to N-N in the ligand (at 1,042 cm^{-1}) was shifted to lower frequency (1,017-1,028 cm^{-1}) in complexes [22]. This shift indicates that bonding in the complexes occurred through the nitrogen atom.

In the low-frequency region, two bands were observed for complexes at ~ 445 and 460 cm^{-1}, which were attributed to υ(M-N) and υ(M-O), respectively. These bands were not found in the spectra of the ligand, suggesting that coordination of the ligand with the metal ions takes place via the azomethine nitrogen atoms and also via the deprotonated phenolic oxygen [21,28].

The characteristic frequencies of the ferrocenyl moiety in the spectra of the ligand were observed at about 3,078, 1,412, 1,111, 1,005, 817, and 490 cm^{-1}. The band at 3,078 cm^{-1} was assigned to the C-H stretching band. The band at 1,412 cm^{-1} was assigned to the asymmetric C-C stretching band. The 1,111 cm^{-1} band was due to asymmetric ring-breathing vibration. The two bands located at 1,005 and 817 cm^{-1} were assigned to parallel and perpendicular C-H bands, respectively. The final band at 490 cm^{-1} was assigned to the Fe-cyclopentadienyl stretching frequency [21-23]. The corresponding frequencies of the complexes appeared at nearly the same position, which indicates that the cyclopentadienyl ring of the ferrocene is not directly coordinated to the metal ion [21-23].

^{1}H-NMR Spectra

NMR spectra of the complexes were recorded in DMSO-d_6 at room temperature using tetramethylsilane (TMS) as internal standard. The

protons in 2/5- and 3/4-position on the cyclopentadienyl rings showed two multiplets at 4.65 and 4.39 ppm [21-23,27]. The signals of the methyl groups were observed at 2.21 ppm in the ligand. These signals were shifted slightly downfield in the spectra of the two complexes, which may be due to complexation of the azomethine nitrogen atoms with the metal ion.

The signal observed for the OH protons of the ligand (ca. 9.72 ppm) was not observed in any of the complexes, which confirms the bonding of the phenolic oxygen to the metal ions (C-O-M) [21, 28]. The signals of the phenyl protons in the ligand and also in the complexes were found in the expected regions at ~ 7.59-6.82 ppm.

Electronic Spectra

The electronic spectra of the complexes were recorded in distilled DMSO. Three d-d transition bands are observed in the regions 510-515, 402-410, and 335-347 nm. These bands are attributed to the $^1A_{1g} \rightarrow {}^1A_{2g}$, $^1A_{1g} \rightarrow {}^1B_{1g}$, and $^1A_{1g} \rightarrow {}^1E_{1g}$ transitions, respectively. The electronic spectra of these complexes indicate the square planar geometry [28-33].

A weak, broad band was also observed for the complexes at 445-451 nm. This band was assigned to the transition $^1A_{1g} \rightarrow {}^1E_{1g}$ in the iron atom of the ferrocenyl group, which indicates that there is no magnetic interaction between the Pd(II), Pt(II) ions, and the Fe(II) ion of the ferrocenyl group [21,22,27].

Based on the spectral data of the complexes discussed above, one can assume that the metal ions are bonded to the ligand via the nitrogen and the phenolic oxygen atom in the two complexes (Fig. 2).

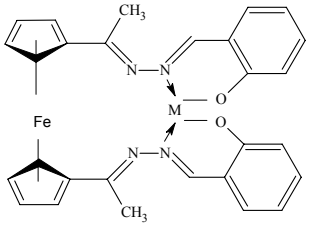

Fig. 2: Structure of the complexes, M = Pd(II) or Pt(II).

Antitumor Activity of the Complexes

Complexes were screened in vitro on MCF-7 human breast cancer cells. Cells exposed to different concentrations of the tested compounds exhibited a dose-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), revealing concentration-dependent increase of inhibition of cell growth (Fig. 3). The doses of the tested compounds were selected based on the preliminary studies.

The tumor cell line showed normal growth in our culture system. DMSO did not seem to have any noticeable effect on cellular growth. A gradual decrease in viability of cancer cells was observed with increasing concentration of the tested compounds, in a dose-dependent inhibitory effect. The Pt(II) complex was more active than the Pd(II) complex, exerting a higher cytotoxicity on MCF-7 cells than the reference compound cisplatin.

The median growth inhibitory concentration (IC_{50}) after 24 h was 17.50 μg/cm^3 for the ligand 12.50 μg/cm^3 for Pd(II) complex, 7.80 μg/cm^3 for Pt(II) complex.

To elucidate the mechanism by which the prepared complexes exert their antitumor activities, we estimated the activities of the free-radical-metabolizing enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as the levels of glutathione (GSH) and H_2O_2 in MCF-7 cells. As shown in Table-1, treatment of the cells with the ligand and its complexes increased the activity of SOD and the level of H_2O_2 (in dose-dependent manner) as compared with the control. In addition, our results revealed that treatment with the complexes leads to decrease in the activity of CAT and GSH-Px as well as the level of GSH (in dose-dependent manner).

The activity was found for both complexes, which resulted in the high SOD activity and H_2O_2 concentration and low activities of CAT and GSH-Px as well as GSH level. The results showed that the antitumor activity of the Pt(II) complex was higher than that of Pd(II) complex. These results indicate that the antitumor effect of the present complexes may be exerted at least partly by production of H_2O_2.

The mode of action of the complexes is still unclear. The antitumor activities are accompanied by dose-dependent increases in SOD activities of treated cells compared with the control group. This means that complexes can cause H_2O_2 production. The H_2O_2 produced should be rapidly removed through the activation of CAT and GSH-Px. The present results show that activities of CAT and GSH-Px and the level of reduced GSH are lowered in groups treated with the complexes (in dose-dependent manner) compared with the control group (Table-1).

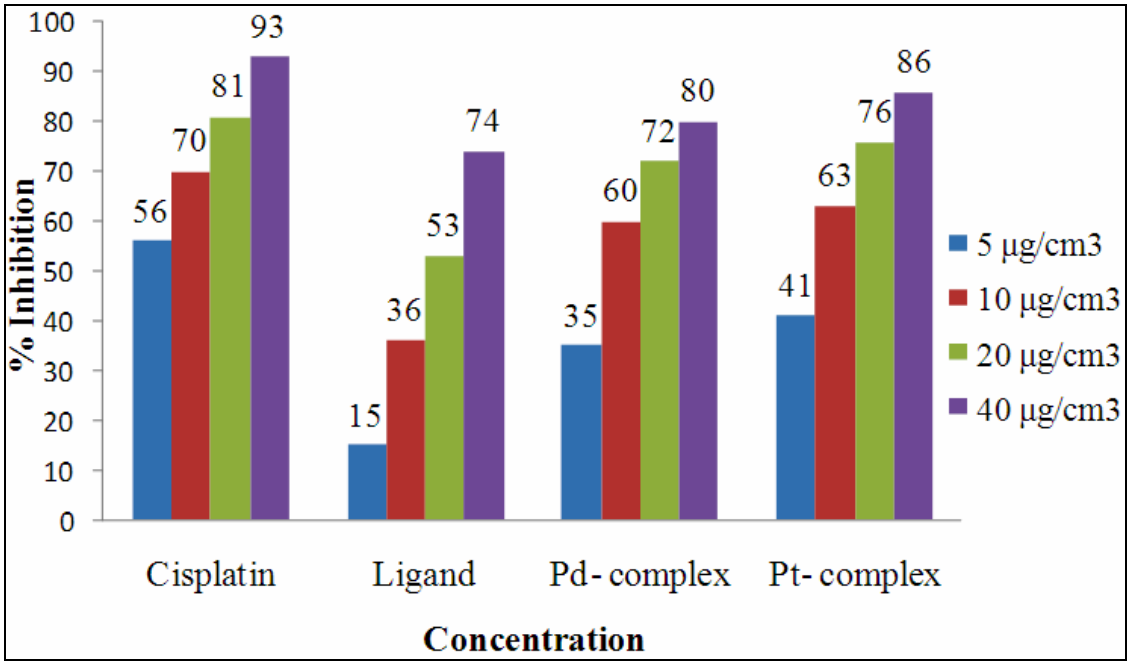

Fig. 3: Effect of different concentrations of the compounds on MCF-7 cell growth, measured by MTT assay.

Table-1: Effect of treatment with different concentrations of the ligand, Pd(II) and Pt(II) complexes on the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) as well as the levels of reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) in MCF-7 cells.

Treatment ($\mu g/cm^3$)	SOD (U/mg protein)	CAT (U/mg protein)	GSH-Px (U/mg protein)	GSH (nmol/mg protein)	H_2O_2 (nmol/mg protein)
Control	30.45 ± 4.11	8.80 ± 0.50	9.50 ± 0.70	37.70 ± 3.20	11.70 ± 1.60
Cisplatin					
5	120.85 ± 13.40	3.11 ± 0.40	4.30 ± 0.48	16.90 ± 1.85	33.60 ± 3.61
10	140.33 ± 15.00	2.80 ± 0.30	3.43 ± 0.39	15.70 ± 1.75	55.20 ± 5.80
20	360.60 ± 24.80	2.44 ± 0.25	2.16 ± 0.25	15.11 ± 2.00	67.75 ± 7.11
40	410.80 ± 35.89	1.50 ± 0.11	1.60 ± 0.2	13.20 ± 1.80	80.50 ± 7.60
Ligand					
5	52.50 ± 2.32	5.00 ± 0.20	6.50 ± 0.49	23.50 ± 2.31	20.54 ± 1.70
10	85.12 ± 5.42	4.04 ± 0.46	6.12 ± 0.53	22.66 ± 2.15	33.82 ± 3.61
20	114.76 ± 9.11	3.25 ± 0.15	5.23 ± 0.39	19.24 ± 1.93	38.23 ± 3.57
40	223.74 ± 21.03	2.78 ± 0.31	4.42 ± 0.42	17.40 ± 1.86	43.91 ± 3.11
Pd(II) complex					
5	85.93 ± 6.03	3.85 ± 0.12	5.11 ± 0.62	21.80 ± 0.24	26.32 ± 2.40
10	122.37 ± 10.42	3.35 ± 0.07	4.91 ± 0.55	19.12 ± 0.33	48.67 ± 2.11
20	318.46 ± 10.63	2.74 ± 0.38	3.68 ± 0.43	17.70 ± 0.23	52.13 ± 3.16
40	360.50 ± 26.40	1.83 ± 0.13	2.28 ± 0.05	15.90 ± 0.21	70.23 ± 4.17
Pt(II) complex					
5	98.90 ± 5.94	3.25 ± 0.20	4.51 ± 0.52	20.27 ± 0.82	28.35 ± 3.91
10	132.32 ± 8.52	2.84 ± 0.42	3.83 ± 0.50	18.19 ± 0.24	51.16 ± 4.77
20	337.84 ± 18.11	2.60 ± 0.15	2.55 ± 0.48	15.41 ± 0.14	62.35 ± 5.31
40	384.51 ± 27.19	1.65 ± 0.13	1.92 ± 0.20	13.89 ± 1.68	78.33 ± 5.26

Data are expressed as mean ± standard error (SE) of six separate experiments. All values for ligand, complexes and cisplatin were significantly different at $p < 0.05$ versus control

Consequently, the excess H_2O_2 produced in tumor cells with complexes cannot be removed. In other words, the accumulation of H_2O_2 in tumor cells should be partly the cause of tumor cell death. Thus the results of the present study are consistent with the hypothesis that the prepared complexes exert their antitumor effects through production of reactive oxygen species (ROS).

The previous results were confirmed by the fact that most chemotherapeutic agents cause cells to over-generate ROS [34], so they are capable of inducing apoptosis, and oxidative damage to DNA, proteins, and lipids. The cascade of signals mediating apoptosis often involves a ROS intermediate messenger, and ROS can short-circuit the pathway, bypassing the need for upstream signals for cell suicide. Recently, Huang *et al.* [35] observed that selective inhibition of SOD kills human cancer cells but not normal cells, suggesting that regulation of free-radical-producing agents may also have important clinical applications. This mechanism for

the effects of ROS-generating anticancer agents is only beginning to be understood, as previously the mechanism of most anticancer agents was believed to be due mainly to direct interaction with DNA and interference with DNA regulatory machinery (e.g., topoisomerases, helicases) and to initiation of DNA damage via production of ROS [36].

Moreover the Pt(II) complex showed the higher antitumor activity, and this can be explained by the fact that platinum is an essential trace element, has important biological functions, especially as antitumor agent [37]. Pd(II) can be reported as promising cytotoxic and antimicrobial agents [38].

Experimental

All chemicals and solvents (AR) were obtained from Merck. 1,1`-diacetyl-ferrocene was prepared according to Rosenblum and Woodward method [39], and 1,1`-diacetylferrocene dihydrazone was synthesized as described by Abd-Elzaher *et al.* [22]. Yields refer to analytically pure compounds and were not optimized. ^{1}H NMR was recorded on Perkin Elmer 283B and 300 MHz Varian XL-300 instruments in DMSO-d_6 as solvent. IR spectra were recorded on a Perkin Elmer (Spectrum 1000) Fourier-transform infrared (FT-IR) spectrometer, using KBr pellets. Elemental analyses were determined at the College of Science, King Saud University, and the results are in agreement with calculated values. Electronic absorptions were recorded on a Shimadzu UV-240 automatic spectrophotometer in DMSO.

1,1`-Bis[1-[2-[(2-hydroxyphenyl)methylene]-hydrazinylidene]-ethyl]-ferrocene ($C_{28}H_{26}FeN_4O_2$) [506.3514]

Salicylaldehyde (2.30 cm^3, 22 mmol) was slowly added to a magnetically stirred solution of 2.98 g 1,1`-diacetylferrocene dihydrazone (10 mmol) in 50 cm^3 methanol. The mixture was refluxed for 8 h. Concentration of the solution to the appropriate volume and cooling to 5 ºC yielded the ligand. The solid was filtered off, washed with cold methanol, and dried. Yield 72%; IR (KBr): ύ = 3,425 (O-H), 1,287 (C-O), 1,615 (C=N), 1,042 (N-N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.21 (s, 6H, 2CH_3), 4.38 (m, 4H, C_5H_4), 4.65 (m, 4H, C_5H_4), 7.56-6.82 (m, 8H, Ph), 8.61 (s, 2H, CH=N), 9.72 (s, 2H, OH) ppm. Calcd for C, H, N: 66.42, 5.17, 11.07; found: 66.21, 5.10, 11.39.

General Procedure for Synthesis of Complexes

Complexes were prepared by addition of 2.0 mmol of anhydrous Palladium (II) chloride (59%-Merck) soluble in 20 cm^3 ethanol and 2.0 mmol Platinum (II) chloride in 20 cm^3 of 1:1 mixture of water and ethanol to a warmed solution of 2.0 mmol of the ligand in 50 cm^3 ethanol. The mixture was refluxed for 3 h. The complex precipitated on cooling to 5ºC, was filtered off, washed two times with cold ethanol, and dried.

[(Ferrocene-1,1`-diyl)bis[2-[(2-ethylidenhydrazinyliden-$_K$N2)-methyl-phenolato]palladium ($C_{28}H_{24}FeN_4O_2Pd$) [610.7574]

Yield 82%; IR (KBr): ύ = 3,417 (O-H), 1, 603 (C=N), 1,305 (C-O), 1,017 (N-N), 460 (M-O), 446 (M-N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.19 (s, 6H, 2CH_3), 4.41 (m, 4H, C_5H_4), 4.72 (m, 4H, C_5H_4), 7.58-6.84 (m,8H, Ph), 8.53 (s, 2H, CH=N) ppm; UV-Vis (MeOH): λmax = 510, 402, 335 nm. Calcd for C, H, N: 55.18, 4.00, 9.17; found: 54.98, 4.19, 9.19.

[(Ferrocene-1,1`-diyl)bis[2-[(2-ethylidenhydrazinyliden-$_K$N2)-methyl]-phenolato]platinum ($C_{28}H_{24}FeN_4O_2Pt$) [649.4554]

Yield 75%; IR (KBr): ύ = 3,410 (O-H), 1,593 (C=N), 1,311 (C-O), 1,028 (N-N), 460 (M-O), 445 (M-N) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.22 (s, 6H, 2CH_3), 4.36 (m, 4H, C_5H_4), 4.70 (m, 4H, C_5H_4), 7.61-6.84 (m,8H, Ph), 8.64 (s, 2H, CH=N) ppm; UV-Vis (MeOH): λ_{max} = 514, 408, 342 nm. Calcd for C, H, N: 51.78, 3.72, 8.63; found: 51.61, 3.77, 8.55.

Cell Culture

The human breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal calf serum (GIBCO), penicillin (100 U/cm^3), and streptomycin (100 µg/cm^3) at 37 ºC in humidified atmosphere containing 5% CO_2. Cells at concentration of 0.50 x 10^6 were grown in a 25 cm^3 flask in 5 cm^3 complete culture medium.

Estimation of in vitro tumor cell growth inhibition was assessed by incubating 0.65 x 10^5 MCF-7 cells in 1 cm^3 phosphate buffer saline with varying concentrations of complexes and cisplatin (as a positive control drug) at 37 ºC for 24 h in CO_2 atmosphere. Cells were cultured for 36 h to ensure total attachment. Afterwards, the tested compounds were added to the cells. Cell survival was evaluated at the end of the incubation period by MTT colorimetric assay. In all cellular experiments results were compared with untreated cells.

Cytotoxicity Assay

The effect of complexes on growth of MCF-7 cells was estimated by MTT colorimetric assay [40]. This method is based on the selective ability of living cells to reduce the yellow soluble salt of MTT to a purple-blue insoluble formazan precipitate. The number of viable cells is proportional to the production of formazan salts. The crystals of formazan were dissolved in DMSO, and the optical density was measured spectrophotometrically (Microplates reader; Asys Hitech, Austria). Cells (0.65×10^5 cells/well) were plated separately in a sterile flat-bottomed 96-well microplate (Falcon) and treated with 30 mm^3 of different concentration of complexes and cisplatin (5, 10, 20, or 40 $\mu g/cm^3$) for 24 h at 37ºC in a humidified 5% CO_2 atmosphere. Then, incubation media were removed and 40 mm^3 MTT solution/well was added and incubated for an additional 6 h. MTT crystals were solubilized by adding 200 mm^3 DMSO/well, and the plate was shaken gently for 10 min at room temperature. The results were determined photometrically using a microplate enzyme-linked immunosorbent assay (ELISA) reader and absorbance at 570 nm. Data are expressed as percentage relative viability compared with untreated cells, calculated using the following equation:

(Absorbance of treated cells) / (Absorbance of control cells) x 100

The cytotoxic concentration was expressed by half maximal IC_{50}. The IC_{50} calculations were performed using Microsoft Excel and Microcal Origin software for PC.

Antioxidants Status Assay

Enzyme activities and the level of both reduced glutathione (GSH) and lipid peroxidation (LP) were expressed in cell lysates as a function of total cellular protein [41]. Activities of SOD, CAT, and GSH-Px were determined as described in literature [42-44]. Levels of reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) were determined using the methods of Ellman [45] and Wolf [46].

Statistical Analysis

The results are reported as mean ± standard error (SE) for at least six experiments. Statistical differences were analyzed using one-way analysis of variance (ANOVA) test followed by t-test, wherein differences were considered significant at $p < 0.05$.

Conclusion

The present results suggest that the prepared complexes possess significant antitumor activity, comparable to the activity of the commonly used anticancer drug cisplatin. These complexes may exert their antitumor activities partly by increasing hydrogen peroxide production and by depletion of intracellular catalase, glutathione peroxidase, and reduced glutathione. The results revealed that these complexes may be potent anticancer agents for inclusion in modern clinical trials.

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