CYTOTOXIC ACTIVITY STUDIES OF SOME PLATINUM(II) COMPLEXES WITH 2-SUBSTITUTEDBENZIMIDAZOLE LIGANDS

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In the present study, two Pt(II) complexes with 2-substitutedbenzimidazole ligands were synthesized and evaluated for their *in vitro* cytotoxic activities on HeLa and MCF-7 cell lines. 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test results showed that complex 2 which was found to be the more active than carboplatin was the most cytotoxic active compound against the HeLa and MCF-7 cell lines among the tested compounds.

INTRODUCTION

Platinum-based drugs are an important cytotoxic drugs used in treatment of a variety of human cancers for more than 50 years.¹ The discovery of the cytotoxic and antitumor properties of cisplatin (cis-diamminedichloroplatinum(II)), was followed by oxaliplatin (*trans*-L-(diaminecyclohexane) oxalateplatinum(II)) in 1979 and carboplatin (cis-diamine(1,1-cyclobutanedicarboxylate)platinum(II)) in 1980 and triggered off the formation of a group of active compounds demonstrating high efficiency toward a number of human tumors.² However, the intrinsic along with acquired resistance and side effects observed in some patients represent major limitations of the treatment of human tumors with the platinum drugs currently used in the clinic.³

It is generally believed that biological activities of cisplatin and its derivatives oxaliplatin and carboplatin are associated with the recognition of its DNA adducts⁴ by cellular proteins such as repair enzymes, transcription factors, histones, and

high mobility group (HMG) domain proteins. Platinum complexes with of action different from that of cisplatin are great interest, because they have the potential to bypass cellular resistance and to present different or fewer toxic side effects.⁵ Design of new platinum antitumor drugs has been introduced on the basis of preventing resistance by enhancing this mechanism using more hydrophobic platinum compounds.⁶

Metal complexes containing bonded ligands having aromatic side arms for use intercalators can be described as dual-function complexes: the aromatic side arms of the ligands can intercalate between DNA bases, whereas the metal coordinates directly to a DNA base. In addition, inclusion of 4-chloro substituent on the typ ligand increase cytotoxic.⁷ Pt(II) complexes bearing conjugated heterocyclic ligands such as benzimidazole and its derivatives constitute a wide class of potential anticancer agents tested in recent years.⁸ There are numerous examples of the substituents on position 2 of benzimidazole carrier ligands of Pt(II) complexes that have been reported

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in literature as efficient DNA intercalation⁹ and active antitumor agents.^{10,11} Gümüş et al. have been investigated a large series of Pt(II) type complexes on *in vitro* cytotoxic against many different human cancer cell lines. It was determined that some of these platinum complexes have *in vitro* cytotoxic activities on human rhabdomyosarcoma (RD)¹², cervix (HeLa)¹³⁻¹⁷, larynx carcinoma (Hep-2)¹³, MDA-MB-231^{16,18,19}, HRT-18²⁰, ovarian cancer (OVCAR)¹⁷ and breast cancer (MCF-7)^{14,15,16,21,22} cell lines.

Fig. 1 – Synthesized platinum (II) complexes.

We use this information in the present study, two Pt(II) complexes with 2-phenylbenzimidazole and 4-chlorophenylbenzimidazole carrier ligands were synthesized and evaluated for their *in vitro* cytotoxic activities on HeLa and MCF-7 cell lines. MTT test results showed that complex 2 which was found to be more active than carboplatin was the most cytotoxic active compound against the HeLa and MCF-7 cell lines among the tested compounds.

RESULTS AND DISCUSSION

The carrier ligands of the complexes synthesized, L¹ and L², were prepared according to the published procedures.²³ Their melting points were in accordance with the literature.^{24,25}

Platinum(II) complex 1 was synthesized as described previously by the reaction of L¹ and K₂PtCl₄ in ethanol/ water solution. IR and ¹H-NMR spectral data of the complex 1 was in accordance with the data reported previously.^{12,26} Platinum(II) complex 2 was synthesized by the reaction of L² and K₂PtCl₄ in DMF solution. The structure of the complex 2 was evaluated using their spectral data, such as IR, ¹H-NMR and ESI-LC/MS, and elemental analysis results. Compounds 1 and 2 were tested for their *in vitro* cytotoxic activities on HeLa, MCF-7 cell lines for the first time.

The complexes were characterized by their elemental analyses and IR and ¹H-NMR spectra compared with those of ligands. Elemental analysis suggested a 1:2 (metal:ligand) stoichiometry for all the complexes as expected. The melting points of the complexes were above 300 °C.

In IR spectrum of L, a very broad band was seen in the region of 3100–2530 cm⁻¹ due to the imidazole N-H. The complexes 1-2 exhibited ν (N-H) stretching bands centered at 3255–3160 cm⁻¹ sharper than that of free ligand due breaking of tautomerism, indicating that the N-H group was not involved in the coordination.^{27,28} The Pt-N and Pt-Cl vibrations are considered to be characteristic for dichloro-diamine platinum complexes. But the metal-nitrogen stretching bands could not be distinguished from other ring skeleton vibrations present in the spectra. In the far-IR region of the complexes 1 and 2 spectra, a new broad band with a half-width of about 30 cm⁻¹ appeared, assigned to ν (Pt-Cl) centered at ~320 cm⁻¹. It is well known that *cis*-dichloro complexes should show two bands of medium intensity because the vibrations are additive, but in a lot of cases the second band is only a shoulder.²⁹

The insolubility of the complexes in the other organic solvents made it necessary to record ¹H-NMR spectra in DMSO-d₆. All ¹H-NMR measurements were recorded immediately in order to avoid the ligand exchange reaction between the platinum complexes synthesized and DMSO-d₆. The spectra of the complexes compared to those of free ligands showed considerable difference. The large downfield shifts in imidazole N1-H signal in the spectra of the complexes with respect to their ligands are a result of an increase in the N1-H acid character after platinum binding.³⁰ The chemical shift variation observed upon coordination (Δδ = δ complex – δ free ligand) shows that most of the values are positive, indicating a decrease in electronic density on the 2-substituted-benzimidazole ligands with coordination to the platinum. The NH signal appear at around 12 ppm in the benzimidazole L and moves downfield in its complexes (13.2–13.4 ppm).

Cytotoxicity. The cytotoxic activities of the platinum complexes 1 and 2, cisplatin and carboplatin were evaluated on the MCF-7 and HeLa cell lines using the MTT cell proliferation assay after 24 h, 48 h and 72 h incubation. The cytotoxic activity values of the platinum complexes 1 and 2 and the reference compounds expressed as Inhibition% are presented in Table 1.

Table 1

Cytotoxicity of compounds 1, 2, cisplatin, and carboplatin on the MCF-7 and HeLa cell lines

Comp.	Inhibition %				Comp.	Inhibition %			
	C (μM)	MCF-7				C (μM)	HeLa		
		24h	48h	72h			24h	48h	72h
1	10	0.92±0.1	0.10±0.02	0.10±0.3	1	10	0.8±0.001	1.10±0.002	1.0±0.003
	20	0.51±0.11	2.0±0.24	2.74±0.41		20	4.36±0.10	3.20±0.22	1.60±0.49
	40	0.85±0.35	1.0±0.32	4.32±0.15		40	4.68±0.96	1.83±2.10	2.35±0.88
	80	2.28±0.26	1.24±0.57	0.63±0.48		80	7.88±0.91	10.27±0.87	11.38±1.21
	160	3.53±0.15	1.45±0.33	9.38±0.86		160	10.06±1.50	13.64±2.20	14.15±2.50
2	10	0.10±0.001	3.35±0.002	1.54±0.003	2	10	2.95±0.51	1.80±0.72	2.0±0.63
	20	2.34±0.10	5.11±0.45	4.73±0.49		20	7.07±0.99 ^a	5.67±1.02	7.50±1.0 ^a
	40	5.69±0.96	16.12±1.49	19.01 ^{a,b} ±0.88		40	12.92±1.0 ^a	21.92±1.29 ^{a,b}	27.45±0.99 ^{a,b}
	80	12 ^{a,b} .22±0.22	39.98 ^{a,b} ±0.97	38.96 ^{a,b} ±1.21		80	50.87 ^{a,b,c} ±1.35	69.12±2.44 ^{a,b,c}	71.03±1.78 ^{a,b,c}
	160	21.24 ^{a,b,c} ±0.93	39.43 ^{a,b} ±1.80	43.91±1.59		160	66.25±1.05	79.45±1.20	82.12±1.99
Cis	5	4.74±0.31	2.48±0.22	20.96±0.53	Cis	5	5.0±0.23	16.10±0.52	21.67±0.70
	10	2.49±0.11	8.86±0.22	32.48±0.61		10	8.30±0.99	18.81±1.10	30.0±1.78
	20	16.76±0.26	25.69±0.20	48.55±0.50		20	46.48±0.91	58.57±0.87	67.02±1.21
	40	42.73±0.66	57.94±0.70	67.48±0.68		40	50.0±1.50	73.91±2.20	80.10±2.50
Carbo	10	0.10±0.001	1.10±0.002	4.68±0.003	Carbo	10	0.7±0.31	1.54±0.22	1.08±0.53
	20	0.57±0.23	1.23±0.52	9.23±0.70		20	0.8±0.11	2.03±0.2	1.3±0.5
	40	0.57±0.99	2.24±1.10	3.89±1.78		40	2±0.26	4.07±0.22	18.79±0.61
	80	6.57±0.81	10.78±0.67	23.67±0.98		80	2.5±0.66	8.33±0.7	26.40±0.8
	160	8.79±1.50	14.35±1.20	39.13±1.50		160	8.51±0.5	14.07±0.2	48.17±0.5

The results (mean $\pm$ standard error) of three independent experiments. a: statistically significant from 10 μ M group ($p \leq 0.05$), b: statistically significant from 20 μ M group ($p \leq 0.05$), c: statistically significant from 40 μ M group ($p \leq 0.05$), d: statistically significant from 80 μ M group ($p \leq 0.05$).

Results are expressed as mean values $\pm$ standard error.

The result of the preliminary cytotoxic activity studies indicated that in general, the following order of relative *in vitro* cytotoxic activity of the compounds tested with HeLa and MCF-7 cell lines could be considered, cisplatin > **2** > carboplatin > **1**.

The cytotoxic activity studies revealed that complex **2** at 81.7 μM concentration causes inhibition approximately 50 % of the HeLa cell population (Table 2).

Table 2

IC₅₀ values for compound **2**, cisplatin, and carboplatin on the MCF-7 and HeLa cell lines

	IC ₅₀ (24 h) (μM)		
	Comp 2	Cis	Carbo
MCF-7	234.9	42.3	310.2
HeLa	81.7	34.5	341.6

It has been reported in the literature³¹ that halogen bonding interactions seem to become comparable in magnitude to the well-studied hydrogen bonds and are responsible for the different conformation of the molecules in the active site. It has also been reported that halogen bonding interactions are one of the important interactions leading to the overall protein-ligand binding affinity. At this stage of the study, it is not possible to explain clearly why complex **2** was the most cytotoxic compound in the cell lines used, compared to the complex **1** tested. However, considering the literature data mentioned above and other data reporting that DNA platination is a necessary condition for the cytotoxic activity of platinum complexes but may not be the sole mechanism that determines their cytotoxicity³², it may be concluded with great caution that chloro substituents on the benzimidazole carrier ligands of complex **2** may play a role in its cytotoxicity. Further experiments are required to verify this conclusion.

EXPERIMENTAL

Chemistry

Materials

All chemicals and solvents used were of reagent grade (Merck, Aldrich, Sigma) and were used without further purification. All cell culture reagents were purchased from Gibco life Technologies, USA.

Melting points were measured on a Electrothermal 9200 melting point apparatus and are uncorrected. Infrared (IR) spectra were obtained using a Perkin Elmer Spectrum 400 FTIR/FTNIR spectrometer equipment with a Universal ATR

Sampling Accessory in the range of 4000–400 cm^{-1} . For the region 400–200 cm^{-1} the samples were performed using FTIR IFS 66/S spectrometer. Elemental analysis were performed with a Leco 932 CHNS analyser, and Proton Magnetic Resonance (¹H-NMR) spectra were recorded in dimethylsulfoxide (DMSO)-d₆ (Merck) on Varian Mercury 400 MHz FT NMR spectrometer. High Resolution Mass Spectra data (HRMS) were collected in-house using a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) operating in either ESI (+) methods, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA). Thin-layer chromatography (TLC) were performed on precoated aluminum plates (Silicagel 60 F₂₅₄, Merck). Plates were visualized by ultraviolet light, Dragendorff reagent, iodine vapor.

General procedure for the synthesis of the ligands

2-phenyl/p-chlorophenylbenzimidazole ligands (L¹/L²) used as ‘carrier ligands’ in the structure of Pt(II) complexes were synthesized by addition of the corresponding aromatic benzaldehydes with the sodium metabisulfide. The solution of p-substituted benzaldehydes in ethanol equimolar sodium metabisulfide was added slowly and the work-up was completed. The 2-substitutedbenzimidazoles were prepared by reaction of o-phenylenediamines with bisulfide addition products in N,N-dimethylformamide. The solution was heated and refluxed for 3 h and subsequently cooled. The crude product was filtered and washed with cold water and crystallized from the alcohol.²³ The melting points of L¹ (found m.p. : 292 °C Lit. m.p. : 293–294 °C)²⁴, L² (found m.p. : 281 °C Lit. m.p. : 285 °C)²⁵ were in accordance with the literature.

Synthesis of the Platinum(II) Complexes

Complex 1

The complex **1** which were synthesized by the other researchers previously were synthesized by the methods as reported before.^{12,26} A solution of L¹ (0.002 mol) was added into a stirred aqueous solution K₂PtCl₄ (0.0011 mol) in ethanol-water mixture by dropwise over 1 h at room temperature. The reaction mixture protected from light was heated at 60 °C for 7 days. After that time the mixture was cooled to room temperature. The resulting precipitate was filtered off, washed several times with small portions of water, ethanol and diethylether and dried *in vacuo*. The yield of purified cis-[PtL₂Cl₂] was 54%, 0.326 g pure. ¹H-NMR (DMSO-d₆): δ (ppm) 13.8 (s, 2H, 2x N-H, exchangeable with D₂O), 9.05–8.89 (m, 8H, ArH), 8.42–8.28 (m, 5H, ArH), 7.91–6.9 (m, 5H, ArH), IR (KBr): ν 3255–3195 (N-H, O-H) cm^{-1} , 328 (Pt-Cl) cm^{-1} . MS (ESI +) m/z: 653.33 [M-H]⁺, calcd. 653.44, 583.47 [M-2Cl]⁺, calcd. 583.54. Anal. Calcd. for C₂₆H₂₀Cl₂N₄Pt. 1.5 H₂O: C, 45.82; H, 3.40; N, 8.22. Found: C, 45.99; H, 3.35; N, 8.06.

Complex 2

228.5 mg (0.001 mol) L² and 207 mg (0.001 mol) K₂PtCl₄ were dissolved in 5 mL DMF. The reaction mixture, protected from light, was heated at 60 °C for 7 days. Precipitated KCl was removed by filtration. A solution of 5 % aqueous KCl (3 mL) was then added to the filtered solution and the mixture was stirred for 2 h. The resulting precipitate was filtered off, washed several times with small portions of water, ethanol and diethylether and dried *in vacuo*. The yield of purified cis-[PtL₂Cl₂] was 12%, 0.080 g pure. ¹H-NMR (DMSO-d₆): 13.5 (broad s, 2H, 2XN-H, exchangeable with D₂O), 9.1 (d, j = 8.8 Hz, 4H, Ar-H), 8.5(d, j = 8.4 Hz, 4H, Ar-H), 8.2–7.4 (m, 8H, Ar-H). C₂₆H₁₈Cl₄N₄Pt.4H₂O: calc. C 39.26, H 3.29, N 7.04; found: C 39.23, H 2.83, N 7.48 %.

IR (KBr) ν 320 (Pt-Cl) cm^{-1} . MS (ESI +) m/z : calcd. m/z 723.33, found m/z 723.34.

Biological Test

The human MCF-7 breast cancer and HeLa cervix cancer cell lines were obtained from Cell Culture Collection HUKUK (Foot and Mouth Disease Institute, Ankara, Turkey). The cells were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM, HyClone Laboratories, Inc., Logan, UT) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria) and 1% penicillin/streptomycin 37 °C and 5% CO₂ in a humidified incubator. Cells were passed every 3 days using trypsin/EDTA solution.

MTT cytotoxicity assay

Cells were suspended in DMEM seeded into 96-well microculture plates (3×10^3 cells/well), and incubated for 24 h, 48 h and 72 h. After 24 h, the medium was aspirated and replaced with serial dilutions of three test compounds (10, 20, 40, 80, 160 μM). Cisplatin and carboplatin were used as reference compounds. At the end of the incubation times, 0.5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT, Sigma, St. Louis, MO, USA) in serum free DMEM was added to each well. The MTT solution was aspirated after a 4 h incubation period, and there sulting purple formazan crystals were dissolved with a 3:80 v/v mixture of ammonia and DMSO. The absorbance in each well optical density was recorded with a microplate reader (Bio Tek Instruments Inc., Winooski, VT) at 550 nm. Experiments have been triplicated. The cell viability was estimated from MTT assay, and the percentage of growth inhibition was calculated using the formula:

$$\text{Percentage viability} = \frac{\text{Mean OD of samples} \times 100}{\text{Mean OD of control group}}$$

The 50% growth inhibitory concentration (IC₅₀) values were calculated by interpolation of the resulting curves.

Statistical analysis

A statistical analysis was performed using the STATISTICA program for Windows. Data were expressed as mean $\pm$ standard error (SE) and statistical significance was assigned at the $P \leq 0.05$ level. Data were analysed using Tukey's post hoc test.

CONCLUSION

In general, the complexes, which were found to be less cytotoxic than cisplatin and were comparable to carboplatin. Compound 2 was more active than the reference compound carboplatin and compound 1 against HeLa and MCF-7 cancer cell lines.

The preliminary data obtained in this study lead us to conclude that the complex with a p-chlorophenyl group on position 2 of the benzimidazole carrier ligand (complex 2) produced appreciable activity against the cell lines used and must be taken into consideration for further

studies. The possible role of the halogen atoms on the cytotoxic activity of the 2-substituted benzimidazole-Pt(II) complexes will also be taken into consideration for future studies.

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