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Original article

Synthesis, cytotoxic activity on MCF-7 cell line and mutagenic activity of platinum(II) complexes with 2-substituted benzimidazole ligands

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Abstract

Four Pt(II) complexes with 2-H/or-methyl/or-aminomethylbenzimidazole or 1,2-dimethylbenzimidazole ligands as ''non-leaving groups'' were synthesized and their antiproliferative properties were tested against the human MCF-7 breast cancer cell line. The mutagenic potentials of the complexes were tested in *Salmonella typhimurium* strains TA 98 and TA 100 in the absence of S9 rat liver fraction. In general, Pt(II) complexes tested which were found to be less active than cisplatin, exhibited moderate in vitro cytotoxic activity on MCF-7 cell line. Among the complexes tested, Pt(II) complex with 2-aminomethylbenzimidazole ligand was found to be highly mutagenic in *S. typhimurium* TA 98 and low mutagenic in *S. typhimurium* TA 100. Pt(II) complex with 1,2-dimethylbenzimidazole was mutagenic only in *S. typhimurium* TA 98. The other two complexes were found to be non-mutagen in both strains.

Keywords: Benzimidazole; Pt(II) complex; Cytotoxic activity; Mutagenic activity

1. Introduction

Cisplatin (*cis*-diaminedichloroplatinum(II)) is one of the most effective drugs in the treatment of testicular, ovarian, bladder, head and neck cancers [1]. Despite the great success of treating certain kinds of cancers, there are several side effects, and both intrinsic and acquired resistances limit the organotropic profile of the drug [2].

There is a continuing interest in the development of new platinum complexes, which are less toxic and non-cross-resistance to cisplatin.

Second-generation platinum(II) antitumour complexes that carry ''non-leaving ligands'' other than simply ammonia are of interest for their ability to modulate drug metabolism and target binding through steric and electronic effects on the substitution mechanism [3]. Several platinum complexes with N-heterocyclic ligands such as imidazole, thiazole, benzimidazole, benzoxazole, and benzothiazole have been reported [4–13]. Some of these platinum complexes showed significant cytotoxicity [4,5,8–13].

The use of amines more compatible to the human system might be another way of surmounting these problems. For this purpose, naturally occurring substances like amino acids, peptides, and glucosamines [14] whose uptake is increased in malignant cells [15] were used as ''non-leaving ligands'' in some Pt-complexes. Some amino acid complexes of Pt(II) exhibited certain antitumour properties [14]. The rational of the synthesis of this type of compounds is the likelihood for facile and selective uptake of the Pt complexes which contain amino acids or sugars as ligands because these natural products are normally taken up by the cell [16,17].

Another approach to the design of novel Pt drugs is to target the Pt coordination moiety to DNA by attaching

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it to a suitable bioactive "non-leaving ligand" including doxorubicin, oestrogen analogues, acridine derivatives, chloroquine, and ethidium bromide. However, to date, there have been no clinically significant advances that have developed from this design approach, and these studies have yielded interesting results, and there is potential for varying the biological activity of these compounds by altering the structure of the "non-leaving ligand" [18].

We have chosen some benzimidazole derivatives as "non-leaving ligands", since the benzimidazole nucleus is found in a variety of naturally occurring compounds such as vitamin B_{12} and its derivatives, and it is structurally similar to purine bases. Furthermore, benzimidazole is known to exhibit a wide variety of pharmacological properties including antitumour activity [19] and inhibition of nucleic acid synthesis [20].

Although there is some evidence to suggest that other biological targets may be important in the cisplatin mechanism, it is generally accepted that DNA is the primary target [2]. The predominant lesions produced in DNA are 1,2 d(GpG) (65%) and 1,2 (ApG) (25%) intrastrand adducts [21]. At this cisplatin-cross-linked site, DNA is strongly kinked and a hydrophobic notch is created which seems to be a target for the binding of damage recognition proteins [22,23]. High-mobility group (HMG) domain proteins are attracted especially by the hydrophobic surface and might mediate the antitumour activity of cisplatin [23,24].

In a previous paper, we have reported the synthesis and characterization of the complexes of the structure, *cis*-$[Pt(L)_2Cl_2]\cdot H_2O$ where L is 5(6)-non/or-chlorosubstituted-2-hydroxymethylbenzimidazole and the determination of their preliminary in vitro cytotoxic effects by "Rec-Assay" test [9]. DNA-binding properties of these two Pt(II) complexes were also examined and it was determined that DNA platinated with these compounds was specifically recognized by HMG domain protein HMG1 [10]. It was concluded that the adducts formed by the compounds distort DNA in a manner similar to cisplatin diadducts. These results encouraged us to design the new benzimidazole Pt(II) complexes.

Deactivation by thiols such as glutathione is considered to be one of the major resistance mechanisms for platinum compounds, together with improved repair of DNA adducts and increased tolerance of DNA damage [25].

The rational design of a platinum drug possessing a reduced susceptibility to binding to thiols would offer the potential to lessen the impact of this mechanism of resistance [26].

Although the presence of bulky, planar amine ligands in *cis*- or *trans*-$[Pt(anion)_2]$ complexes and their orientation with respect to the coordination plane, as well as their substituents, can reduce the rates of DNA binding or thio binding compared to aliphatic amine and amine complexes [27], creating the more hydrophobic area around the drug-DNA binding site by these amine ligands than cisplatin may increase the binding of HMG domain proteins to the widened and kinked DNA and can shield the major 1,2-intrastrand adducts from the access of the nucleotide excision-repair (NER) complex [28].

In this study, benzimidazole ligands having hydrogen, methyl or aminomethyl groups at position 2 were chosen as the "non-leaving ligands" of the Pt(II) complexes in consideration of three main reasons. The sterically hindering ligands may reduce rapid detoxification by thiol-containing molecules. The probability of the use of bidentate ligand such as 2-aminomethylbenzimidazole instead of simple NH_3 may prevent translabilization and undesired displacement of the "non-leaving ligand" by sulphur and nitrogen donors and also hydrophobic properties of the ligands was taken into consideration.

In this paper, four Pt(II) complexes with the ligands 2-H/or-methyl/or-aminomethylbenzimidazole and 1,2-dimethylbenzimidazole were synthesized and evaluated for their preliminary in vitro cytotoxic activities on the human MCF-7 breast cancer cell line and for their mutagenic activities.

2. Results and discussion

2.1. Synthesis and characterization of the complexes

Four mono- or disubstituted benzimidazole derivatives used as "non-leaving ligands" in the structure of the Pt(II) complexes (**1**–**4**) were prepared according to Phillips method [29] as shown in Fig. 1 and their melting points were in accordance with the literature [30–32]. The Pt(II) complexes were synthesized by the reaction of the ligands with K_2PtCl_4 in ethanol–water solution. The melting points of all complexes were above 400 °C.

The complexes obtained were characterized by their elemental analyses and IR and ^{1}H-NMR spectra. Although, the ligands synthesized in this study were reported previously by other researchers for the characterization of the complexes by comparison with the data of the ligand, IR and ^{1}H-NMR data of the ligands were also performed. It was not possible to obtain suitable crystals for structural determinations. However, from the results of the techniques employed it is possible to propose the structures. Elemental analyses suggested a 1:2 (metal:ligand) stoichiometry for Pt(II) complexes **1**, **2**, and **4** and 1:1 stoichiometry for **3**.

IR spectrum of the complexes has shown some characteristic changes when compared to the free ligands. The ligands except Ambim.2HCL show broad bands in the region 3300–2300 cm^{-1} due to the intermolecular hydrogen bonded imidazole N–H. All the complexes which have free imidazole N–H were exhibited N–H stretching bands ranging from 3270 to 3215 cm^{-1} sharper than those of the ligands due to

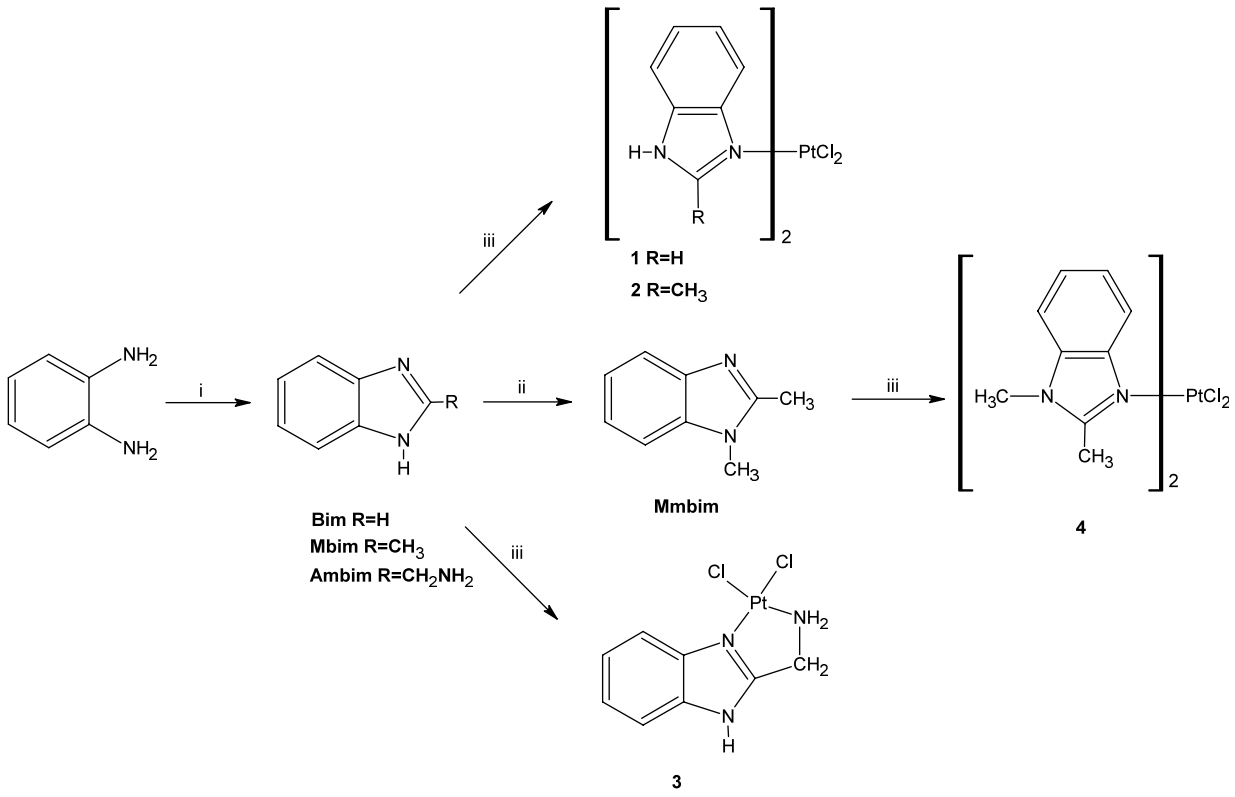

Fig. 1. Synthesis of ligands and their Pt(II) complexes. Reagents: (i) RCOOH, 5N HCl, reflux 2–30 h (ii) NaH, MeI, DMF, reflux 24 h (iii) K_2PtCl_4, $EtOH/H_2O$, t.

breaking of tautomerism, indicating that imidazole N–H was not involved in the coordination [30,33].

The Pt–N and Pt–Cl vibrations are considered to be characteristic for dichlorodiamine platinum complexes. But except complex **3**, for the other three complexes metal–nitrogen stretching bands could not be distinguished from the other ring skeleton vibrations present in the spectra. For compound **3**, the broad new band centred at 260 cm^{-1} assigned to ν(Pt–N) vibration. According to the kinetic *trans* effect [34], the synthesis method used is expected to yield complexes with *cis* geometry. In the far-IR region of the spectra of all the complexes appears a new band assigned to ν(Pt–Cl) centred around 327–315 cm^{-1} characteristic for *cis*-configurated dichloro-Pt(II) complexes [35].

The other bands in the spectrum of each complex were similar to those in the corresponding ligand spectrum except for slight shifts in their positions and changes in their intensities due to coordination.

The insolubility of the complexes in the other organic solvents made it necessary to record ^{1}H-NMR spectra in dimethylsulfoxide-d_6 (DMSO-d_6). All ^{1}H-NMR measurements were recorded immediately in order to avoid the ligand exchange reactions between the Pt(II) complexes and DMSO-d_6.

^{1}H-NMR spectral data of the ligands and their Pt(II) complexes are presented in Table 1. ^{1}H-NMR spectrum of the Pt(II) complexes in DMSO-d_6 is indicative of complex formation. The spectra of the complexes compared to those of the free ligands showed considerable difference. The large downfield shift in the imidazole N–H signal in the spectra of all complexes respect to their ligands are a result of an increase in the N–H acid character after platinum binding [36]. The chemical shift variation upon coordination ($\Delta\delta = \delta$ complex $- \delta$ free ligand) observed show that most of the values are positive, indicating a decrease in electronic density on the 2-substituted benzimidazole ligands with coordination to platinum.

For complex **4** with 1,2-dimethybenzimidazole ligand, *syn*- and *anti*-rotamers were observed in ^{1}H-NMR in the ratio 1:1.

2.2. Biological activities

The preliminary antiproliferative activity of the platinum(II) complexes bearing 2- or 1- and 2-substituted benzimidazole ligands as "non-leaving groups" were determined on the human MCF-7 breast cancer cell line. MCF-7 cells were incubated for 48, 72, and 96 h with 0.5, 1, 5, 10, and 20 μM benzimidazole Pt(II) complexes and cisplatin as reference. The antiproliferative activity values of the complexes and the reference cisplatin expressed as $T/C_{corr.}$ are presented in Fig. 2.

At a dosage of 0.5 and 1 μM, no statistically significant cytotoxicity was observed for all the compounds synthesized. At these concentrations, the refer-

Table 1
^{1}H-NMR data [a] of the ligands and their Pt(II) complexes

Compound	CH_3	CH_2	NH_3^+/NH_2	N–H	$N–CH_3$	Ar–H
Bim	–	–	–	12.40 (br, 1H)	–	8.19 (s, 1H), 7.59 (dd, $J = 8.5$ and 3.2 Hz, 2H), 7.19 (dd, $J = 9.5$ and 3.2 Hz, 2H)
1	–	–	–	13.46 (s, 2H)	–	8.83 (s, 2H), 7.81 (d, $J = 7.3$ Hz, 2H), 7.51 (d, $J = 7.1$ Hz, 2H), 7.23 (m, 4H)
Mbim	2.48 (s, 3H)	–	–	12.25 (brs, 1H)	–	7.44 (dd, $J = 9.0$ and 3.2 Hz, 2H), 7.09 (dd, $J = 9.2$ and 3.2 Hz, 2H)
2	[b]	–	–	13.18 (brs, 2H)	–	8.05–7.18 (m, 8H)
Ambim·2HCl	–	4.51 (s, 2H)	9.10 (s, 3H)	[b]	–	7.78 (dd, $J = 9.3$ and 3.2 Hz, 2H), 7.45 (dd, $J = 6.1$ and 3.1 Hz, 2H)
3	–	4.05 (t, $J = 6.0$ Hz, 2H)	6.09 (brt, $J = 6.0$ Hz, 2H)	13.55 (s, 1H)	–	8.56 (d, $J = 7.7$ Hz, 1H), 7.59 (d, $J = 7.7$ Hz, 1H), 7.50–7.21 (m, 2H)
Mmbim	2.52 (s, 3H)	–	–	–	3.72 (s, 3H)	7.50 (d, $J = 7.3$ Hz, 1H), 7.45 (dd, $J = 7.5$ Hz, 1H), 7.15 (dtd, $J = 7.5$, 6.04 and 1.2 Hz, 2H)
4	3.01 and 2.88 (2s, 3H each)	–	–	–	3.79 and 3.76 (2s, 3H each)	8.19–8.16 (m, 2H), 7.57–7.53 (m, 2H), 7.44–7.26 (m, 4H)

[a] The spectra were recorded at 400 MHz as DMSO-d_6 solutions with TMS as internal standard δ (ppm).
[b] Obscured by the solvent signals.

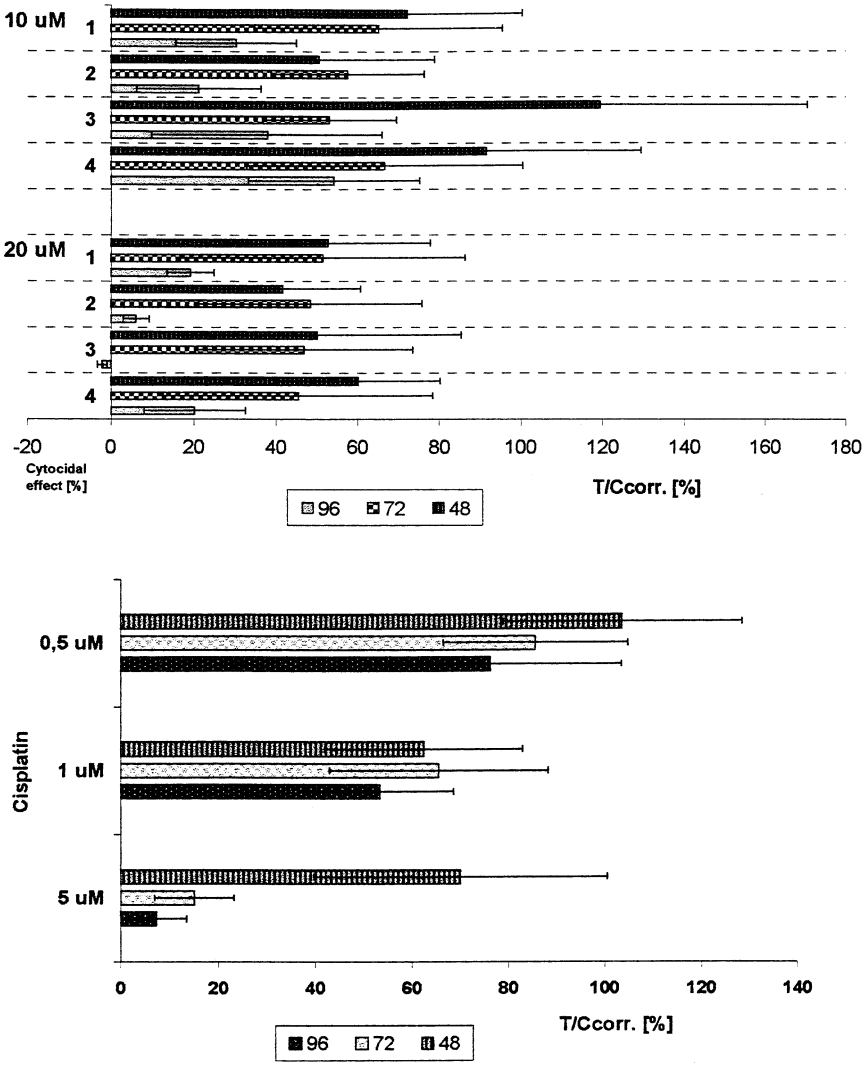

Fig. 2. Effect of the benzimidazole–platinum complexes (**1**–**4**) and the reference cisplatin on the proliferation of MCF-7 breast cancer cell line.

ence cisplatin had $T/C_{corr.}$ values of ca. 76 and 53%, respectively, after the incubation period of 96 h (Fig. 2).

At 5 μM concentration, cytotoxic effects of complexes **1** and **2** amounted to $T/C_{corr.}$ values of ca. 77 and 73%, respectively, at the 96 h time point (data not shown). While at the same concentration and time point, $T/C_{corr.}$ value of cisplatin was around 7% (Fig. 2).

At 10 μM concentration among the synthesized complexes, complexes **1** and **2** bearing Bim and Mbim, respectively, as "non-leaving ligands" were found to be the most active compounds with $T/C_{corr.}$ values of around 30 and 21%, respectively, at the last time point.

A clear antiproliferative effect was observed for all the complexes synthesized by increasing the concentration to 20 μM. At this dosage, $T/C_{corr.}$ values of complexes **1**–**4** were around 19, 6, −2 (cytocidal effect), 20%, respectively, at the 96 h time point.

Although complex **2** with the ligands having free N–H moiety in its structure was found to be slightly more active than its corresponding methylated derivatives (complex **4**) to confirm the role of free N–H group of the benzimidazole ligands on the cytotoxic activity of the benzimidazole–platinum(II) complexes, further chemical and biological activity studies are required.

The predominant lesions produced in DNA by cisplatin are 1,2 d(GpG) (65%) and 1,2 (ApG) (25%) intrastrand adducts [21]. These are believed to be responsible for not only the cytotoxicity but also the mutagenicity of cisplatin [37]. Mutagenicity may represent a serious limitation to the therapeutic use of potential drugs. This adverse property should be eliminated by rational drug design when possible.

The mutagenic potential of the Pt(II) complexes synthesized was investigated by short-term bacterial mutagenicity in reverse-mutation assay using *Salmonella typhimurium* frameshift strain TA 98 and *S. typhimurium* TA 100, which carry mutations particularly sensitive to reversion by DNA base pair substitution. The aim of the mutagenic activity studies was to eliminate the complexes in which the mutagenic potency governs for further antitumour activity studies. The tests were performed in the absence of S9 rat liver fraction. Cisplatin was tested for comparison. The direct-acting mutagenicity results of the compounds are summarized in Table 2.

Complex **3** with Ambim ligand which caused an increasing 7-, 14-, 18-, and 10-fold in the number of revertants at 10, 25, 50, and 100 μg per plate, respectively, was found to be highly mutagenic in TA 98. This complex caused a dose-related increase at concentrations 10, 25, and 50 μg per plate in TA 100. Complexes **1** and **2** were found to be non-mutagenic in TA 98 and TA 100 in the range of dosages tested. Cisplatin showed mutagenic response at extremely low doses (1 μg per plate) in TA 100 and very low level of frameshift reversion in TA 98 at concentration 5 μg per plate. Complex **4**, *N*-methyl derivative of complex **2**, caused an increasing twofold in the number of revertants at 10 μg per plate in TA 98.

It is possible to suggest that the presence of aminomethyl group is likely to be the major factor in the mutagenicity of the platinum(II) complex **3**. TA 98, sensitive to frameshifts, was found to be more sensitive to complex **3** than was TA 100. These results were in agreement with the reports in the literature on the mutagenicity of some benzimidazole derivatives [19,38,39] that the nature of the side groups on the benzimidazole ring greatly influenced the mutagenic activity of the benzimidazole derivatives.

3. Conclusion

Pt(II) complexes tested in this study, which were found to be less active than cisplatin, exhibited moderate cytotoxic activity on the human MCF-7 breast cancer cell line. Although among the compounds tested Pt(II) complexes with 2-aminomethylbenzimidazole ligand was the most active one, this complex was also found to be highly mutagenic. In conclusion, complexes **1** and **2**, which were found to be non-mutagen and are supposed to have potential advantages in terms of reduced side effects, warrant further investigation into their in vitro and in vivo antitumour activity against different types of tumours. And also, requirement of the confirmation of non-mutagenicity of these complexes with additional testing will be taken into consideration.

4. Experimental

4.1. Chemistry

4.1.1. Materials

Melting points were measured on a Electrothermal 9200 melting point apparatus and are uncorrected. Elemental analyses were performed by TÜBITAK Laboratory (Ankara, Türkiye). Infrared (IR) spectra were recorded in KBr pellets and in Nujol mulls on a Mattson 1000 FTIR spectrometer in the range 4000–200 cm^{-1}. For the region 400–200 cm^{-1}, the samples were prepared as Nujol mulls on CsI windows. Proton magnetic resonance (^{1}H-NMR) spectra were recorded in DMSO-d_6 (Merck) on a Bruker 400 MHz spectrometer. All chemicals and solvents used were of reagent grade (Merck, Aldrich, Sigma), and were used without further purification. Thin-layer chromatography (TLC) was performed on pre-coated aluminium plates (Silica gel 60 F_{254}, Merck). Plates were visualized by UV light, Dragendorff reagent, iodine vapour.

4.1.2. Synthesis of platinum(II) complexes

*4.1.2.1. cis-[Dichloro-bis(benzimidazole)platinum(II)]·[Pt(Bim)$_2$Cl$_2$] (**1**).* To a stirred solution of Bim (0.216 g, 2 mmol) in ethanol–water mixture (5–20 mL) was

Table 2
The mutagenic activities of the platinum(II) complexes in Ames test system with *S. typhimurium* TA 98 and TA 100

Compound	Dose (μg per plate)	Revertant colony numbers	
		TA 98 mean ± S.D., $n = 10$	TA 100 mean ± S.D., $n = 10$
1	0	43 ± 5.6	114.3 ± 25.5
	10	42.5 ± 6	129 ± 31.6
	25	43.1 ± 6.5	143.8 ± 21.9
	50	47.6 ± 7.4	152 ± 35.7 [a]
	100	42.3 ± 14.2	162 ± 37.2 [a]
	F	0.665	3.718
	P	0.620	0.011
2	0	13.1 ± 3	92.4 ± 13.8
	10	10.9 ± 4.4	86.9 ± 12.1
	25	9.8 ± 4.9	80.7 ± 10.3
	50	11.1 ± 2.8	80.3 ± 16.1
	100	8.1 ± 2.5 [a]	72.7 ± 12.2 [c]
	150	6.5 ± 4.4 [a]	85 ± 29.5
	F	2.883	2.091
	P	0.024	0.083
3	0	39.3 ± 10.3	111.3 ± 14.5
	10	339.1 ± 118.4 [b]	164.1 ± 32.5 [b]
	25	544 ± 125.7 [b]	217.5 ± 25.7 [b]
	50	738.2 ± 100.4 [b]	270.4 ± 32.8 [b]
	100	421.3 ± 215.7 [b]	140.3 ± 62.7
	F	38.730	29.041
	P	0.000	0.000
4	0	15.3 ± 2.4	121.9 ± 41.5
	10	34.6 ± 7.4 [a]	171.9 ± 25.6 [a]
	25	19.6 ± 7.2	109.6 ± 23.7
	50	15.8 ± 3.2	95.8 ± 26.6
	100	20.2 ± 7.4	101.4 ± 20.7
	F	17.45	11.454
	P	0.000	0.000
		$n = 8$	$n = 8$
Cisplatin	0	20.2 ± 1.6	81 ± 11
	1	31.6 ± 5.7 [a]	195.2 ± 40 [a]
	2.5	33.2 ± 8.3 [a]	269 ± 54.1 [a]
	5	39.5 ± 7.9 [a]	222.5 ± 44.8 [a]
	10	30.1 ± 7.2	98.4 ± 62.3
	15	15.5 ± 4.6	33.2 ± 21.4
	F	9.225	24
	P	0.000	0.000

[a] $P < 0.05$ for Dunnett's t-test.
[b] $P < 0.05$ for Dunnett's C-test.
[c] $P < 0.1$ for Dunnett's t-test.

added an aqueous solution of K_2PtCl_4 (0.415 g, 1 mmol in 5 mL H_2O) dropwise over 30 min at room temperature. The pH was adjusted to ~8 and kept constant with the addition of 0.1 M KOH. The reaction mixture protected from light was heated at 60 °C for 1 day. The mixture was cooled to 0 °C. The resulting precipitate was filtered off, washed several times with small portions of water, ethanol, and diethylether and dried in vacuo. Yield: 98%, 0.419 g pure. Anal. Calc. for $C_{14}H_{12}Cl_2N_4Pt$: C, 33.47; H, 2.40; N, 11.15. Found: C, 32.78; H, 2.42; N, 10.75%. IR (KBr): 3270 (N–H), 1609 (C=N), 744 (=C–H), 327 (Pt–Cl) cm^{-1}.

*4.1.2.2. cis-[Dichloro-bis(2-methylbenzimidazole)-platinum(II)] [Pt(Mbim)$_2$Cl$_2$] (**2**).* A similar procedure was carried out using Mbim (0.264 g, 2 mmol) and K_2PtCl_4 (0.415 g, 1 mmol) at 60 °C for 12 days. Yield: 76%, 0.460 g pure. Anal. Calc. for $C_{16}H_{16}Cl_2N_4Pt$: C, 36.23; H, 3.04; N, 10.56. Found: C, 36.49; H, 3.98; N, 9.49%. IR (KBr): 3264 (N–H), 1620 (C=N), 748 (=C–H), 320 (Pt–Cl) cm^{-1}.

*4.1.2.3. cis-[Dichloro-(2-aminomethylbenzimidazole)-platinum(II)] [Pt(Ambim)$_2$Cl$_2$] (**3**).* A similar procedure was carried out using Ambim·2HCl (0.280 g, 0.5

mmol), $NaHCO_3$ (0.084 g, 1 mmol), and K_2PtCl_4 (0.252 g, 0.6 mmol) at room temperature for 2 days. Yield: 74%, 0.154 g pure. Anal. Calc. for $C_8H_9Cl_2N_3Pt$: C, 25.25; H, 2.19; N, 10.17. Found: C, 25.19; H, 2.45; N, 9.84%. IR (KBr): 3215 (N–H), 1620 (C=N), 771 (=C–H), 327 (Pt–Cl), 315 (Pt–Cl) cm^{-1}.

*4.1.2.4. cis-[Dichloro-bis(1,2-dimethylbenzimidazole)-platinum(II)] [Pt(Mmbim)$_2$Cl$_2$] (**4**).* A similar procedure was carried out using Mmbim (0.292 g, 2 mmol) and K_2PtCl_4 (0.415 g, 1 mmol) at 60 °C for 10 days. Yield: 44%, 0.256 g pure. Anal. Calc. for $C_{18}H_{20}Cl_2N_4Pt$: C, 38.71; H, 3.61; N, 10.03. Found: C, 37.94; H, 3.43; N, 9.51%. IR (KBr): 1615 (C=N), 764 (=C–H), 322 (Pt–Cl) cm^{-1}.

4.2. Biological tests

4.2.1. Preliminary cytotoxicity test

4.2.1.1. Cell line and growth conditions. The human MCF-7 breast cancer cell line used in this study was obtained from the Cell Culture Collection (HUKUK No. 00092502) of Institute for Foot and Mouth Disease (IFMD) (Türkiye).

The cells were grown in Dulbecco's (Seromed, Germany) minimal essential medium (DMEM) enriched with 10% foetal calf serum (FCS; Biochrom, Germany), 100 mg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested using trypsin (Bibco Life Technologies, UK)/Versen (0.05%:0.02%) solution. Mycoplasma contamination was routinely monitored and only mycoplasma-free cultures were used.

4.2.1.2. In vitro chemosensitivity assay on the human MCF-7 breast cancer cell line. The preliminary in vitro testing of the platinum complexes on antitumour activity was carried out on human MCF-7 breast cancer cells according to a previously published microtitre test [40]. Briefly, the cells were seeded into 96-well plates (Greiner GmbH, Germany) in a volume of 100 μL as to be 18–22 cells/microscopic area. After attachment to the culture surface, the cells were incubated in an atmosphere containing 5% CO_2 at 37 °C for 24 h. After 48 h, the growth medium was carefully removed by suction and 200 μL of fresh medium were added into each well. The medium used contained an adequate volume of a stock solution of the respective compound in order to obtain the desired test concentration (0.5, 1, 5, 10, and 20 μM, solvent: DMSO, the complexes tested were added to the culture medium such that the final DMSO was 0.1% (v/v)).

16 wells were used for each compound (complexes **1**–**4** and cisplatin) tested for individual concentrations, while 16 wells were reserved for the cell culture control, which contained the corresponding amount of DMSO. After the proper incubation time, the medium was removed and the cells were fixed with 100 μL, 1% glutardialdehyde in phosphate-buffered saline (PBS) per well for 25 min. The fixative was replaced by 150 μL PBS/well and the plates were stored in the refrigerator (4 °C). Cell biomass was determined by a crystal violet staining technique [41].

The effects of the platinum complexes were expressed as corrected T/C values according to the following equations:

$$T/C_{\text{corr.}}(\%) = \frac{T - C_o}{C - C_o} \times 100$$

where T is the mean absorbance of the treated cells, C the mean absorbance of the controls, and C_o the mean absorbance of the cells at the time ($t = 0$) when the drug was added.

When the absorbance of treated cells was less than that of the culture at $t = 0$ (C_o), the extent of cell killing was calculated as

$$\text{Cytocidal effect } (\%) = \frac{C_o - T}{C_o} \times 100$$

Absorbance was measured at 578 nm using a Titertek Multiscan Plus MKII Autoreader.

All platinum(II) complexes were tested in two independent duplicates.

4.2.2. Bacterial mutagenicity assay

4.2.2.1. Material and methods. Chemicals used were obtained from the following sources: sodium azide and D-biotin (Sigma Chemical Co., St. Louis), Daunomicine (Deva Holding A.[a]), L-histidin-HCl monohydrate (BDH), Bacto agar, and Oxoid-nutrient broth No. 2 (Oxoid).

4.2.2.2. Tester strains. S. typhimurium TA 98 (his D3052, rfa, Δuvr B, pKM101) and TA 100 (his G46, Δuvr B, pK_M 101) were kindly provided by Dr. Bruce Ames (University of California, Berkeley, CA). All strains were stored at −80 °C and were routinely checked to ensure the presence of appropriate genetic markers and spontaneous reversion patterns. Sodium azide and daunomicine were used as diagnostic mutagens without metabolically active rat liver S9 fraction for *S. typhimurium* TA 100 and *S. typhimurium* TA 98, respectively.

Overnight growth was initiated with inoculation from master plate into Oxoid-nutrient broth No. 2. Following overnight growth, all tester strains were diluted into the same culture and grown with shaking at 37 °C (ca. 5 h). When cultures reach a density of 0.300 OD at 650 nm 1–2 × 10^9 cells mL^{-1}, they were used in the mutagenicity experiments [42].

4.2.2.3. Cytotoxicity assay. The amounts of test compounds to be used in the mutation assays were selected in cytotoxicity assay. 0.1 mL of a suitable dilution of an overnight bacterial culture was added to 2 mL top agar together with different concentration of the compounds in DMSO. DMSO is one of the compatible organic solvents with Ames test system. It has no toxic and mutagenic effects on the tester strains in the amounts used [43]. The top agar was poured onto nutrient agar plates and assessment of cytotoxicity was made after 24 h incubation at 37 °C [44].

4.2.2.4. Mutagenicity assay. The method used was basically the same as described by Maron and Ames [42]. Briefly 0.1 mL of bacterial tester strain and sample to be tested (in freshly prepared DMSO solution) was added to 2 mL of molten top agar. The contents were mixed and poured on agar plates. After 2–3 days of incubation, revertant colonies were counted. At least 10 plates were used for each dose. The strains were checked routinely for ampicilline resistance UV-light sensitivity, crystal violet sensitivity, histidine requirement, and spontaneous reversion rate. They were stored at −80 °C. In *Salmonella*/microsome test system, data are interpreted on the basis of a consistent doubling of the spontaneous reversion frequency confirmed by a dose–response relationship. Where the number of induced revertants is less than twice the spontaneous rate but a reproducible dose-related increase in revertants is detected, this is also interpreted as a positive response [44]. The concentrations reported were designed to include the highest non-toxic dose.

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References

[1] I.H. Krakoff, in: M. Nicali (Ed.), Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy: Clinical Application of Platinum Complexes, Martinus Nijhoff, Boston, MA, 1988, p. 351.
[2] E.R. Jamieson, S.J. Lippard, Chem. Rev. 99 (1999) 2467–2498.
[3] K. Wang, J. Lu, R. Li, Coord. Chem. Rev. 151 (1996) 53–88.
[4] E.S. Domnina, V.N. Varopayev, G.G. Skvorsov, S.M. Minakova, B.A. Cervou, Khim. Farm. Zh. 17 (6) (1983) 700–703.
[5] S.S. Mylonas, A. Valavanidis, K. Dimitropoulus, M. Polissiouv, A.S. Tsiftsoglov, I.S. Vizirianakis, J. Inorg. Biochem. 34 (1988) 265–275.
[6] M.M. Muir, G.M. Gomez, M.E. Cadiz, J.A. Muir, Inorg. Chim. Acta 168 (1990) 47–57.
[7] M.M. Muir, O. Cox, L.A. Rivera, M.E. Cadiz, E. Medina, Inorg. Chim. Acta 191 (1992) 131–139.
[8] M.J. Bloemink, H. Engelking, S. Karentzopoulos, B. Krebs, J. Reedijk, Inorg. Chem. 35 (1996) 619–627.
[9] F. Gümüş, F. İzgü, Ö. Algül, FABAD J. Pharm. Sci. 21 (1996) 7–15.
[10] F. Gümüş, Ö. Algül, J. Inorg. Biochem. 68 (1997) 71–74.
[11] G. Tallen, C. Mock, S.B. Gangopadhyay, B. Kangarloo, B. Krebs, J.E.A. Wolff, Anticancer Res. 20 (2000) 445–450.
[12] J. Jaley, W.I. Cross, R.G. Pritchard, C.A. McAuliffe, K.B. Nolan, Inorg. Chim. Acta 315 (2001) 36–43.
[13] C. Mock, I. Puscasu, M.J. Rauterkus, G. Tallen, J.E.A. Wolff, B. Krebs, Inorg. Chim. Acta 319 (2001) 109–116.
[14] A. Iakovidis, N. Hadjiliadis, Coor. Chem. Rev. 135/136 (1994) 17–63.
[15] W. Beck, B. Purucker, M. Girnth, H. Schönenberger, H. Seidenberger, G. Ruckdeschel, Z. Naturforsch. 31b (1976) 832–845.
[16] E. Bersanetti, A. Pasini, G. Pezzoni, G. Pratesi, G. Savi, R. Supino, F. Zunino, Inorg. Chim. Acta 93 (1984) 167.
[17] A. Colombo, R. Di Gioia, A. Pasini, T. Dasdia, F. Zunino, Inorg. Chim. Acta 125 (1986) L1.
[18] E. Wong, C.M. Giandomenico, Chem. Rev. 99 (1999) 2451–2455.
[19] A.A. Spasov, I.N. Yozhitsa, L.I. Bugaeva, V.A. Anisimova, Pharm. Chem. J. 33 (1999) 232–243.
[20] R.A. Bucknall, S.B. Carter, Nature (1967) 1099–1101.
[21] A. Eastman, Biochemistry 25 (1986) 3912.
[22] C.A. Bewley, A.M. Gronenborn, G.M. Clore, Ann. Rev. Biophys. Biomol. Struct. 27 (1998) 105–131.
[23] U.M. Ohndorf, M.A. Rould, Q. Ite, C.O. Pabo, S.J. Lippard, Nature 399 (17) (1999) 708–712.
[24] D.B. Zamble, S.J. Lippard, TIBS 20 (1995) 435–439.
[25] R.P. Perez, Eur. J. Cancer 34 (1998) 1535–1542.
[26] L.R. Kelland, S.Y. Sharp, C.F. O'Neill, F.I. Raynaud, P.J. Beale, I.R. Sudson, J. Inorg. Biochem. 77 (1999) 111–115.
[27] J. Reedijk, Chem. Rev. 99 (1999) 2499–2510.
[28] S.J. Brown, P.J. Kellet, S.J. Lippard, Science 261 (5121) (1993) 603–605.
[29] M.A. Phillips, J. Chem. Soc. (1928) 2393–2399.
[30] D.J. Rabiger, M.M. Joullie, J. Org. Chem. 29 (1964) 476–482.
[31] H. Irving, O.A. Weber, J. Chem. Soc. (1959) 2560–2565.
[32] B. Pilarski, Liebigs Ann. Chem. (1983) 1078–1080.
[33] R.C. Maurya, D.D. Mishra, Synth. React. Inorg. Met. Org. Chem. 20 (80) (1990) 1013.
[34] T. Gündüz (Ed.), Koordinasyon Kimyasi, Bilge Yayincilik, Ankara, 1994.
[35] V. Callagan, D.M.L. Goodgame, R.P. Tooze, Inorg. Chim. Acta 78 (1) (1983) 51–54.
[36] B. Lippert, Prog. Inorg. Chem. 37 (1989) 1–94.
[37] L.J.N. Bradley, K.J. Yarema, S.J. Lippard, J.M. Essinman, Biochemistry 32 (1993) 982.
[38] F.T. Hatch, M.E. Colvin, Mutat. Res. 376 (1997) 87–96.
[39] C.J. Goin, V.W. Mayer, Mutat. Res. 343 (1995) 185–199.
[40] G. Bernhardt, H. Reile, H. Birnböck, T. Spruss, H. Schönenberger, J. Cancer Res. Clin. Oncol. 118 (1992) 35–43.
[41] H. Reile, H. Birnböck, G. Bernhardt, T. Spruss, H. Schönenberger, Anal. Biochem. 187 (1990) 262–267.
[42] D.M. Maron, B.N. Ames, Mutat. Res. 113 (1983) 173–215.
[43] D.M. Maron, J. Katzonellenbogen, B.N. Ames, Mutat. Res. 88 (1981) 343–350.
[44] B.J. Dean, T.M. Brooks, G. Hodson-Walker, Mutat. Res. 1153 (1985) 57–77.