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Original article

Synthesis, in vitro cytotoxic and antiviral activity of *cis*-[Pt(R(–) and S(+)-2-α-hydroxybenzylbenzimidazole)$_2$Cl$_2$] complexes

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Abstract

A pair of enantiomeric platinum(II) complexes of *cis*-[Pt(R(–) and S(+)-HBB)$_2$Cl$_2$] (HBB = 2-α-hydroxybenzylbenzimidazole) was synthesized and evaluated for its preliminary in vitro cytotoxic activity on the human MCF-7 breast cancer and HeLa cervix cancer cell lines and antiherpes virus activity against bovine herpesvirus type 1 (BHV-1). In general, it was found that Pt(II) complexes were less cytotoxic on both cell lines than cisplatin and were comparable to carboplatin. There was no significant difference in cytotoxicity between two enantiomers, and the antiviral test results showed that the Pt(II) complexes and their carrier ligands R(–) and S(+) HBB had no effects inhibiting replication of BHV-1.

Keywords: 2-α-Hydroxybenzylbenzimidazole; Pt(II) complexes; Cytotoxic activity; Antiviral activity

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)) is a widely used chemotherapeutic agent for the treatment of testicular cancer and it is used in combination regimens for a variety of other tumors, including ovarian, cervical, bladder, lung and those of the head and neck [1]. Despite the success of cisplatin, problems regarding intrinsic or acquired resistance and side effects have encouraged the development of new platinum drugs. Acquired resistance, as well as the intrinsic resistance observed in some patients, is multifactoral in nature and includes contributions from differential drug uptake, cellular detoxification systems, and DNA repair mechanisms [2,3]. Structural modification of the carrier-ligand in cisplatin may alter the spectrum of antitumor activity and overcome resistance [4].

The carrier-ligand of the platinum DNA-damaging agents may affect biodistribution; rates and type of DNA adduct formation, recognition of damaged DNA by repair enzymes or regulatory/binding proteins [5]. The use of sterically demanding diamines as carrier-ligands as an alternative compound to cisplatin can slow or block repair enzymes [6].

The studies on the search for new platinum drugs revealed that not only the chemical constitution but also the configuration of the carrier-ligand determines the extent of the antitumor effect [7]. Since the chirality of the carrier-ligands determines the tendency of a complex to be transported through the cell membrane [8] and DNA has a chiral structure and its reaction with stereoisomeric diamineplatinum(II) complexes yields adducts which are diastereomeric to each other and thus hinder the DNA function to different extents, the use of optically pure chiral ligands might be an alternative means in order to get better characteristics for platinum compounds.

In a previous paper, we reported the synthesis and characterization of the complexes of the structure, *cis*-[Pt(L$_2$)Cl$_2$]·H$_2$O 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]. The DNA-binding properties of these two Pt(II) complexes were also examined and it was determined that the DNA platinated with these compounds was specifically recognized by high mobility group (HMG) domain protein,

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HMG1 [10]. It was also determined that some of the new 2-substituted benzimidazole Pt(II) complexes we synthesized have in vitro cytotoxic activities on the human RD Rhabdomyosarcoma [11] and MCF-7 breast cancer [12] cell lines.

In the present study to determine whether enantiomorphic properties of the platinum complexes influence their cytotoxic activities, two enantiomerically pure Pt(II) complexes bearing R(–) or S(+)-2-α-hydroxybenzylbenzimidazole (HBB) as carrier-ligands were synthesized and evaluated for their preliminary in vitro cytotoxic activities on the human MCF-7 breast cancer and HeLa cervix cancer cell lines. With the consideration that interference of platinum complexes with viral nucleic acids could be important also in the treatment of viral disease, antiherpesvirus (bovine herpesvirus type 1, BHV-1) activities of the platinum complexes, *cis*-[Pt(R(–)-HBB)$_2$Cl$_2$] and *cis*-[Pt(S(+)-HBB)$_2$Cl$_2$], were also tested.

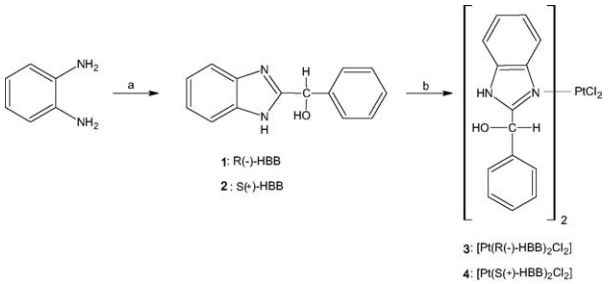
2. Results and discussion

2.1. Synthesis and characterization of the complexes

The carrier-ligands R(–)-HBB and S(+)-HBB (**1** and **2**) in the Pt(II) complexes (**3** and **4**) were prepared starting from commercially available R(–) and S(+) mandelic acid, according to Phillips method [13] as shown in Scheme 1. Their melting points and optical rotation values were identical to the data reported in the literature [14,15]. The Pt(II) complexes **3** and **4** were synthesized by the reaction of enantiomerically pure ligands with K_2PtCl_4 in 0.5 N HCl as described in Section 4. The melting points of the complexes were above 300 °C.

The complexes were characterized by elemental analysis, fast atom bombardment (FAB) mass, infrared (IR) and ^{1}H-NMR spectra. It was not possible to obtain suitable crystals for structural determinations. However, from the results of the techniques employed it is possible to purpose possible structures (Scheme 1). Elemental analysis suggested a 1:2 (metal/ligand) stoichiometry for the complexes.

The IR spectra of the complexes have shown characteristic changes when compared to free ligands. The ligands show broad bands in the region 3530–2300 cm^{-1} due to the intermolecular hydrogen bounded imidazole N–H. The complexes exhibited N–H stretching bands centered at 3170 cm^{-1} sharper than those of the ligands due to breaking of tautomerizm, indicating that the N–H group was not involved in the coordination [16,17]. The complexes showed a broad band centered at 3422 cm^{-1} assigned to υ (OH) which is not appreciably shifted from the free ligand values, suggesting that coordination does not occur through the oxygen.



Scheme 1. (a) R(–) or S(+)-mandelic acid, 4 N HCl, reflux 1–2 days, (b) K_2PtCl_4, 0.5 N HCl, 50 °C.

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. According to the kinetic *trans* effect [18], the synthesis method used is expected to yield complexes with *cis* geometry. In the far IR region of the complexes's spectra, a new broad band appeared assigned to υ (Pt–Cl) centered at 327 cm^{-1}. As 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 [19]. In some cases, *cis*-dichloro complexes show only one band due to low resolutions independent from *cis* or *trans*-configurations [20]. Although no shoulder was apparently observable at the Pt–Cl stretching band of the complexes **3** and **4**, the broad nature of the band suggested the presence of the bands overlapped in this domain. In addition the position of the υ (Pt–Cl) band of the complexes was at 327 cm^{-1} and the geometry can be assigned as *cis*. The other bands in the spectrum of the complexes were similar to those in the ligands 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 the ^{1}H-NMR spectra in dimethylsulfoxide-d_6 (DMSO-d_6). All measurements were recorded immediately in order to avoid the ligand exchange reactions between the Pt(II) complexes and DMSO-d_6.

The ^{1}H-NMR spectrum of the Pt(II) complexes in DMSO-d_6 is indicative of complex formation. The ^{1}H-NMR spectra of R(–)- and S(+)-HBB-platinum(II)complexes **3** and **4** are identical. The spectra of the complexes compared to those of the free ligands showed considerable difference. The large downfield shifts in the imidazole N–H signal in the spectra of the complexes with respect to their ligands are a result of an increase in the N–H acid character after platinum binding [21]. The chemical shift variation upon coordination ($\Delta\delta = \delta$ complex $- \delta$ free ligand) observed, show that some of the values are positive, indicating a decrease in electronic density on the 2-substituted benzimidazole ligands with coordination to the platinum. The ^{1}H-NMR signals of the platinum complexes tend to broaden, this is consistent with the literature [22].

In the FAB MS spectrum of platinum complex **3**, the peak with very low intensity corresponding to its molecular ion, M^+, was shown at *m*/*z* 713.9 (calc. 714.51) while in the spectrum of complex **4** this peak did not appear. Peaks at *m*/*z* 609.6 and 609.5 assignable to fragments corresponding to the loss of Cl^- and OH^- ions of platinum complexes **3** and **4**,

respectively, were observed. The loss of Cl^- ions has been well documented for similar structure of complexes [23].

2.2. Biological activities

2.2.1. Preliminary cytotoxicity test

The preliminary antiproliferative activity of the platinum complexes bearing enantiomeric R(–) or S(+)-HBB groups as carrier-ligands were determined on the human MCF-7 breast cancer and HeLa cervix cancer cell lines. MCF-7 and HeLa cells were incubated for 72 h with 40, 20, 10, 1, 5, 0.5 μM of the Pt(II) complexes and cisplatin and carboplatin used as reference compounds. The antiproliferative activity values of the complexes and the reference compounds expressed as $T/C_{corr.}$ are presented in Table 1.

In the test on the human MCF-7 cancer cell line at 0.5, 1, 5 μM concentration $T/C_{corr.}$ values of the [Pt(R(–)/and S(+)-HBB)Cl_2] (**3** and **4**) except for complex **3** at 5 μM concentration were >100. At these concentrations, the reference cisplatin and carboplatin had $T/C_{corr.}$ values of ca. 82.89%, 66.69%, 10.52% and >100%, >100%, 81.64%, respectively. At 10 μM concentration, cytotoxic effects of the complexes **3** and **4** amounted to $T/C_{corr.}$ values of ca. 57.91% and 80.85%, respectively. While at the same concentration $T/C_{corr.}$ values of the reference compounds cisplatin and carboplatin were found to be –22.44% (cytocidal effect) and 87.79%, respectively.

A clear antiproliferative effect was observed for both complexes synthesized by increasing the concentration to 20 μM. At these dosage $T/C_{corr.}$ values of complexes **3** and **4** were around 12.63% and 16.99%, respectively. At this concentration cytocidal effects were observed for cisplatin and carboplatin (–42.80% and –8.88%, respectively). At the concentration of 40 μM, cytocidal effects were observed for the complexes synthesized and for the reference compounds.

The test results for the platinum(II) complexes on the human HeLa cervix cancer cell line showed that the complexes were less active than cisplatin. Considerable $T/C_{corr.}$ values of the complexes **3** and **4** on HeLa cell line were in between 40 and 20 μM concentration. (Table 2).

In general, it was found that Pt(II) complexes were less cytotoxic on both cell lines than cisplatin and were comparable to carboplatin. Although the complexes bearing R(–)-HBB ligands were found to be slightly more active than the complexes bearing S(+)-HBB ligands on the MCF-7 cell line, no significant differences between the enantiomeric forms of platinum(II) complexes were observable. These results are consistent with previous reported data that the differences in biological activity of enantiomeric forms of platinum(II) compounds with chiral amine ligands are not so pronounced unless the rational freedom of the asymmetric center is reduced by placing, for instance, the chiral center(s) on the carbon chain linking two nitrogen atoms of a chelate diamine [7].

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 [24]. DNA-binding reactions of platinum complexes are usually viewed as kinetically controlled processes [25,26]. Steric hindrance of the carrier-ligands can slow reaction kinetics [27]. The presence of bulky, planar amine ligands in [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 compared to aliphatic ammine and amine complexes [6]. The ligands used in this study are not flate due to the α-hydroxybenzyl moiety. This may result in severe steric constraints around the Pt atom, making the complexes less reactive than cisplatin. For the same reason Pt–Cl hydrolysis of the platinum complexes **3**, **4** may be relatively slow compared to cisplatin. A detailed study

Table 1
Effect of the Pt(II) complexes on the proliferation of MCF-7 cell line

	$[T/C]_{corr.}$	(%) (S.D.) [a]				
Compound	40 μM	20 μM	10 μM	5 μM	1 μM	0.5 μM
Cis-[Pt(R(–)HBB)$_2$Cl$_2$]	–51.55 ± 1.12 [b]	12.63 ± 4.72	57.91 ± 26.89	94.26 ± 2.83	>100	>100
Cis-[Pt(S(+)HBB)$_2$Cl$_2$]	–20.86 ± 4.92 [b]	16.99 ± 1.24	80.85 ± 18.24	>100	>100	>100
Cisplatin	–49.91 ± 6.26 [b]	–42.80 ± 20.47 [b]	–22.44 ± 3.00 [b]	10.52 ± 5.14	66.69 ± 20.15	82.89 ± 4.90
Carboplatin	–9.88 ± 0.25 [b]	–8.88 ± 3.51 [b]	87.79 ± 20.17	81.64 ± 1.31	>100	>100

[a] S.D., standard deviation.
[b] Cytocidal effect.

Table 2
Effect of the Pt(II) complexes on the proliferation of HeLa cell line

	$[T/C]_{corr.}$	(%) (S.D.) [a]				
Compound	40 μM	20 μM	10 μM	5 μM	1 μM	0.5 μM
Cis-[Pt(R(–)HBB)$_2$Cl$_2$]	–5.37 ± 1.01 [b]	55.69 ± 9.95	50.40 ± 4.72	84.66 ± 9.38	>100	>100
Cis-[Pt(S(+)HBB)$_2$Cl$_2$]	8.12 ± 0.50	36.18 ± 8.15	66.22 ± 5.16	71.07 ± 6.89	>100	>100
Cisplatin	–48.60 ± 12.85 [b]	–53.69 ± –14.90 [b]	–58.81 ± 15.68 [b]	–54.43 ± 15.00	13.21 ± 4.85	44.03 ± 4.40
Carboplatin	16.13 ± 2.43	59.37 ± 20.43	76.78 ± 19.22	89.72 ± 11.03	>100	>100

[a] S.D., standard deviation.
[b] Cytocidal effect.

determining the complex binding and hydrolysis rate constants of the complexes will be necessary to investigate these hypotheses. The insolubility of the Pt(II) complexes in water compared to cisplatin may also be related to their lower cytotoxicity.

2.2.2. Antiherpes virus effect tests

Although it is well known that HBB selectively inhibits only certain viruses belonging to the Picornavidae family, while it is completely inactive against the main viruses of other families [15,28], in this study with the aim of evaluating to what extent complexation affects the antiherpesvirus activity of the R(–) and S(+)-HBB ligands, the antiviral activity of the ligands and the complexes against BHV-1 were tested.

In all series of BHV-1 infected Madin-Darby bovine kidney (MDBK) cells incubated with 150, 50 and 20 μM of platinum complexes **3**, **4** and also 10 μM of R(–)-HBB ligand **1**, high toxicity resulted in massive cellular death were found as early as at 8–12 h postinoculation (pi). S(+)-HBB **2** was found extremely toxic for MDBK cells in its all concentrations used in research. The rest of the cells completed their incubation period in parallel to non-treated MDBK cells showing BHV-1 indicative cytopathic changes until 84 h pi (Fig. 1).

Plaque assay revealed no significant difference in plaque numbers and plaque sizes which were detected between the viruses incubated with the platinum complexes and R(–)-HBB ligand (Table 3). Based on the data obtained from plaque assay, one step growth curves of the BHV-1 showed that there are no obvious changes between growth kinetics of the viruses. These results indicated that platinum complexes **3**, **4** and R(–)-HBB ligand have no effects inhibiting replication of BHV-1.

3. Conclusion

The main purpose of this work was to investigate whether differences in cytotoxicity on MCF-7 and HeLa cell lines exist between a pair of enantiomeric complexes, [Pt(R(–)-HBB)$_2$Cl$_2$] and [Pt(S(+)-HBB)$_2$Cl$_2$] and to test their antiherpesvirus type 1 (BHV-1) activities. The test results showed that no significant differences in cytotoxicity between enantiomers were observable and the platinum complexes and their carrier-ligands, R(–) and S(+)-HBB, had no effects inhibiting replication of BHV-1. In general, the complexes, which were found to be less cytotoxic than cisplatin, exhibited moderate cytotoxicity comparable to carboplatin in both MCF-7 and HeLa cell lines. In conclusion, complexes **3** and

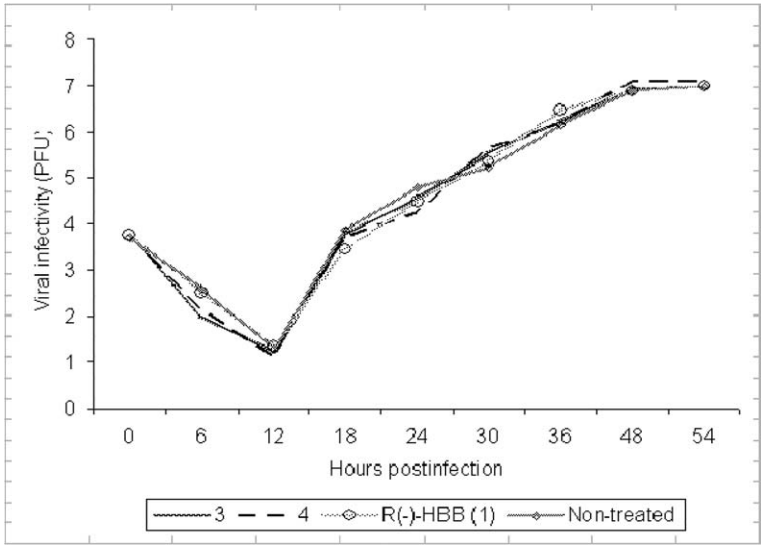


Fig. 1. One step growth curve of BHV-1 in MDBK cells in the presence of Pt(II) complexes **(3)** and **(4)** and ligand R(–)-HBB **(1)**.

Table 3
Average plaque numbers of BHV-1 dilutions treated and untreated with Pt(II) complexes **(3)** and **(4)** and ligand R(–)-HBB **(1)**

Log 10 dilutions	Plaque numbers of BHV-1 after incubation with								
	3			**4**			R(–)-HBB **(1)**		Plain
	10 μM	5 μM	1 μM	10 μM	5 μM	1 μM	5 μM	1 μM	DMEM
1	UC	UC	UC	UC	UC	UC	UC	UC	UC
2	UC	UC	UC	UC	UC	UC	UC	UC	UC
3	UC	UC	UC	UC	UC	UC	UC	UC	UC
4	UC	UC	UC	UC	UC	UC	UC	UC	UC
5	79	83	82	78	81	81	80	91	86
6	11	11	12	10	10	11	11	10	11
7	1	2	2	1	1	1	0	0	2

UC, uncountable.

4 should be considered for further antitumor activity studies within an extended series of structural derivatives and a broader range of cell lines.

4. Experimental

4.1. Chemistry

4.1.1. Materials

Melting points were measured on an Electro thermal 9200 melting point apparatus and are uncorrected. Elemental analyses were performed by TÜBİTAK Laboratory (Ankara, Turkey). IR spectra were recorded on KBr pellets and Nujol mulls on a Mattson 1000 FTIR spectrometer in the range of 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 precoated aluminum plates (Silicagel 60 F_{254}, Merck). Plates were visualized by UV light, Dragendorff reagent, and iodine vapor. Specific rotations were measured with a Rudolph Research Analytical Autoral (IV) polarimeter, at room temperature (20 °C); concentrations are expressed as g per 100 ml. Positive-ion FAB^+ mass spectra were recorded on a ZapSpec spectrometer using 3-nitrobenzyl alcohol (NBA) as the matrix solvent.

4.1.2. Synthesis of R(–) and S(+)-2-α-hydroxybenzylbenzimidazoles. [R(–) and S(+) HBB] (*1* and *2*)

R(–) or S(+)-mandelic acid 2.28 g (15 mmol) and 1,2-phenylendiamine 1.08 g (10 mmol) were dissolved in 10 ml 4 N hydrochloric acid and refluxed for 3 h. The reaction mixture was then allowed to cool to room temperature and placed in a refrigerator (5 °C) for 1 h. A white precipitate, formed, was filtered off and then dissolved in 25 ml water. The clear solution was neutralized with a solution of 20% aqueous solution of K_2CO_3 and a white precipitate which formed, was filtered off, washed with water and recrystallized several times from ethanol/water (1:1). R(–)-HBB **1** yield: 45.86%, 1.03 g pure, S(+)-HBB **2** yield: 43.63%, 0.98 g pure, m.p. 204 °C (ethanol/water (1:1)) (lit. 205–207 °C [29]). ^{1}H-NMR (DMSO-d_6): δ 12.24 (broad s, 1H, N–H), 7.52–7.49 (m, 4H, Ar–H), 7.37–7.34 (m, 2H, Ar–H), 7.29–7.25 (m, 1H, Ar–H), 7.19–7.15 (m, 2H, Ar–H), 6.57 (s, 1H, OH), 5.97 (s, 1H, CH). IR (KBr): υ 3425 (O–H), 3200–3100 (N–H), 1045 (C–O) cm^{-1}.

For evaluation of the specific rotations of the enantiomers, the hydrochlorides of R(–) and S(+) HBB were prepared, since the optical rotation values $[\alpha]_D$ for the base form of the enantiomers in absolute ethanol and acetone were very low [14]. R(–) or S(+) HBB were dissolved in absolute ether and saturated with a stream of dry HCl. The precipitate was filtered off and recrystallized from isopropyl alcohol/ether (1:1).

$[\alpha]_D^{20}$: R(–)-HBB hydrochloride: –82.3° (c = 1, water) (–83.6° (water) [14], –82.7° (water) [15]), S(+)-HBB hydrochloride: +83.7° (c = 1, water) (+84.5° (water) [14], +83.2° (water) [15]), m.p. 210 °C (isopropyl alcohol/ether (1:1)) (210–211 °C [15]).

4.1.3. Synthesis of cis-[Pt(R(–)-HBB)$_2$Cl$_2$] (*3*) and cis-[Pt(S(+)-HBB)$_2$Cl$_2$] (*4*)

To a stirred solution of R(–) or S(+)-HBB (0.45 g, 2 mmol) in 0.5 N HCl (10 ml) was added a solution of K_2PtCl_4 (0.415 g, 1 mmol) in 0.5 N HCl (10 ml) drop wise over 2 h at room temperature. The reaction mixture, protected from light, was heated at 50 °C for 1 or 2 days for R(–) or S(+) enantiomer, respectively. A pink solution and reddish oil was formed during this period. The solution was discarded and the reddish oil was solidified to give a pink precipitate by treating with ethanol/ether mixture (1:1 volume) and stirring for 1 h, which was then collected by filtration, washed with ethanol followed by ether. This procedure was repeated for three times. The product was dried in vacuo.

^{1}H-NMR (DMSO-d_6): δ 13.65 (broad s, 2H, 2 × N–H, exchangeable with D_2O), 7.98–7.54 (m, 4H, Ar–H), 7.39–7.12 (m, 8H, Ar–H), 6.88–6.32 (m, 6H, Ar–H), 6.25 (s, 2H, 2 × O–H, exchangeable with D_2O), 5.71 (s, 2H, 2 × CH), IR (KBr): υ 3170 (N–H), 1036 (C–O), 327 (Pt–Cl) cm^{-1}. *Cis*-[Pt(R(–)-HBB)$_2$Cl$_2$] **3**: yield: 44.8%, 0.320 g pure. FAB-MS(+): m/z 713.9 $[M]^+$, calc. 714.51, 609.6 $[M - 2Cl^- - 2OH^-]^+$, calc. 609.58. Anal. calc. for $C_{28}H_{24}Cl_2N_4O_2Pt$: C, 47.06; H, 3.38; N, 7.84%. Found: C, 46.52; H, 3.78; N, 7.09%. *Cis*-[Pt(S(+)-HBB)$_2$Cl$_2$] **4**: yield: 39.9%, 0.285 g pure. FAB-MS(+): m/z 609.5 $[M - 2Cl^- - 2OH^-]^+$, calc. 609.58. Anal. calc. for $C_{28}H_{24}Cl_2N_4O_2Pt$: C, 47.06; H, 3.38; N, 7.84%. Found: C, 47.22; H, 3.32; N, 7.39%.

4.2. Biological test

4.2.1. Preliminary cytotoxicity test

4.2.1.1. Cell lines and growth conditions. The human MCF-7 breast and HeLa cervix cancer cell lines used in this study was obtained from the Cell Culture Collection (HUKUK No: 00092502 and 90061901, respectively) of Institute for Foot and Mouth Disease (IFMD) (Turkey).

The cells were grown in Dulbecco's (Seromed, Germany) minimal essential medium (DMEM) enriched with 10% fetal 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 and HeLa cervix cancer cell lines. The preliminary in vitro testing of the platinum complexes on

antitumor activity was carried out on human MCF-7 breast and HeLa cervix cancer cells according to a previously published microtiter test [30]. Briefly, the cells were seeded in to 96-well plates (Greiner GmbH, Germany) in a volume of 100 μl as to be 18–22 cells per 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, 20 and 40 μM solvent: DMSO, the complexes tested were added to the culture medium such that the final DMSO was 0.1% (v/v)). Sixteen wells were used for each compound (complex **3**, **4**, cisplatin and carboplatin) tested for individual concentrations while 16 wells were reserved for the cell culture control, which contained the corresponding amount of DMSO. After the 72 h 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 [31].

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

$$\text{T/Ccorr. } [\%] = [(\text{T} - \text{Co})/(\text{C} - \text{Co})] \times 100$$

where *T* is the mean absorbance of the treated cells, *C* is the mean absorbance of the controls, and C_o is 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

$$\text{Cytocidal effect } [\%] = [(\text{Co} - \text{T})/\text{Co}] \times 100$$

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. In vitro antiherpes virus activity tests

4.2.2.1. Virus and cell culture. Cooper strain of BHV-1 used in the research was propagated in MDBK cells with routine procedure. The virus was stored at –80 °C in small aliquots after purification and infectivity titer of the virus was also calculated.

4.2.2.2. Test for antiviral effect. Serial concentrations of R(–) and S(+)-HBB ligands and their platinum complexes **3** or **4** were tested on the BHV-1 infected MDBK cells and the results were compared in terms of plaque size and number detected pi in parallel to non-treated BHV-1. MDBK cells monolayered in tissue culture flasks were infected with BHV-1 Cooper strain at a multiplicity of infection (MOI) of 0.1 plaque morphing unit (PFU) per cell. Cells were incubated at 37 °C for 1 h for providing viral attachment onto cell membrane. After intensive washing of cell surface with pre-warmed PBS, DMEM containing the platinum compounds and ligands (dissolved in DMSO) in final concentrations of 150, 50, 20, 10, 5 and 1 μM were added to culture flasks. One series of cell were incubated with plain DMEM serving as non-treated virus control. The cells infected were further incubated at 37 °C for several days until cytopathic effect (cpe) in cells infected with non-treated BHV-1 has appeared during daily microscopic evaluations. The cultures were frozen and thawed twice after cpe reached 95% of cells. The yields of the virus were aliquoted in small volumes and stored at –80 °C until use.

4.2.2.3. Detection of infectivity titer. To detect in vitro infectivity of the viruses after incubation with the compounds tested, the plaque assay was used. Confluent MDBK cells grown in 24-well plates were infected with 10-fold serial dilutions (0.2 ml) of viruses harvested from cells incubated with/without the compounds. After 1 h adsorption at 37 °C, cells were overlaid with 1.6% carboxymethylcellulose in DMEM. The cells were incubated for further 48 h and then were fixed with 10% formaldehyde for 1 h at room temperature. After removal, fixative cells were stained with crystal violet (0.35%) in ethanol. Plaques formed by per dilution were measured (or counted) and represented as the average of duplicate inoculation wells.

4.2.2.4. One step growth curve. The growth dynamics of the virus after incubation with and without the compounds tested were performed as described by Chowdhury et al. [32]. Briefly, confluent MDBK cells were infected with viruses at a MOI of 5 PFU per cell. After 1 h of adsorption at 4 °C, residual input viruses were removed. The cultures were washed three times with PBS, and 5 ml of medium was added to each flask before further incubation (37 °C). At indicated time intervals, replicate cultures were frozen. Virus yields were determined by titration on MDBK cells. Each data point represented the average of duplicate samples obtained from separate infections.

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