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Half-Sandwich Ru(II) Complexes Bearing 2-(2′-quinoly)benzimidazoles with Anticancer Activity

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Abstract: In this article, cytotoxic and apoptotic properties of previously synthesized and characterized four different half - sandwich ruthenium (II) complexes (**C_1-C_4**) with 2-(2′-quinoly) benzimidazole frameworks (**L_1-L_5**) were described. These Ru (II) complexes (**C_1-C_4**) had strong cytotoxic activity towards the human glioblastoma (U373) cancer cell line with low toxicity to the non-cancerous human embryonic kidney (HEK293) cell line. Mechanistic studies revealed that all complexes caused apoptosis induction by activating caspases with upregulation of *Bax* and downregulation of *Bcl-2*. Our results indicate that ruthenium (II) complexes with 2-(2′-quinoly) benzimidazole frameworks, especially **C_1** and **C_4**, had a higher cytotoxic and apoptotic activity in human glioblastoma cells, and they should be further evaluated in detail for its anticancer properties as a new therapeutical strategy for glioblastoma.

Keywords: 2-(2′-quinoly)benzimidazole; half-sandwich Ru(II); piano-stool complex; anticancer; glioblastoma.

1. Introduction

The approach to treating cancer has changed progressively from conventional chemotherapy to targeted therapies that interfere with crucial signaling pathways within tumors [1]. Glioblastomas (GBMs) constitute 80% of all central nervous system cancers, with glioblastoma multiforme being among the most aggressive variations [2]. Treatment strategies for GBMs are short-lived and have limited effects on overall survival. The current treatment protocol involves surgical removal followed by a combination of radiotherapy and chemotherapy [3]. The prognosis of glioblastoma multiforme (GBM), one of the most common brain neoplasms, is frequently unfavorable. Treatment depends on some factors, such as the age of the patient and the sensitivity of the tumor to chemotherapy. Moreover, tumors commonly recur either during or shortly after the initial treatment, resulting in a median survival period of only 15 months for individuals newly diagnosed with GBM [4].

In the field of inorganic medicinal chemistry research, the design of metal-containing cancer chemotherapeutic agents is in the first place [5,6]. Besides being interesting as catalysts, Ruthenium complexes have emerged as promising antitumor or antimetastatic agents [7,8]. Notably, following the first approved ruthenium(III) complexes, NAMI-A, imidazolium[trans-tetrachloro(1H-imidazole)(dimethylsulfoxide)ruthenate(III)] [9], KP1019, indazolium[trans-tetrachlorobis(1H-indazole)ruthenate(III)], and IT-139, sodium[trans-tetrachlorobis(1H-

indazole)ruthenate(III)] (also known as NKP-1339) [10,11] in clinical trials, Ru(II)-polypyridyl compound (TLD-1433) has recently entered phase IB clinical trials as a photodynamic therapy (PDT) agent in the treatment of bladder cancer, as seen in Figure 1 [12]. The reduction of these complexes to the more reactive Ru(II) analogy is thought to result in effective cytotoxicity in vivo [13]. In addition to these, some important arene-ruthenium(II) complexes (by the Sadler and the Dyson groups) with antineoplastic/antimetastatic activity have also been developed (Figure 1) [14]. After the advent of platinum-containing drugs [15], the tendency of widely applied platinum-based drugs to show undesirable side effects, the curative properties of ruthenium complexes as therapeutic agents are remarkable today [16].

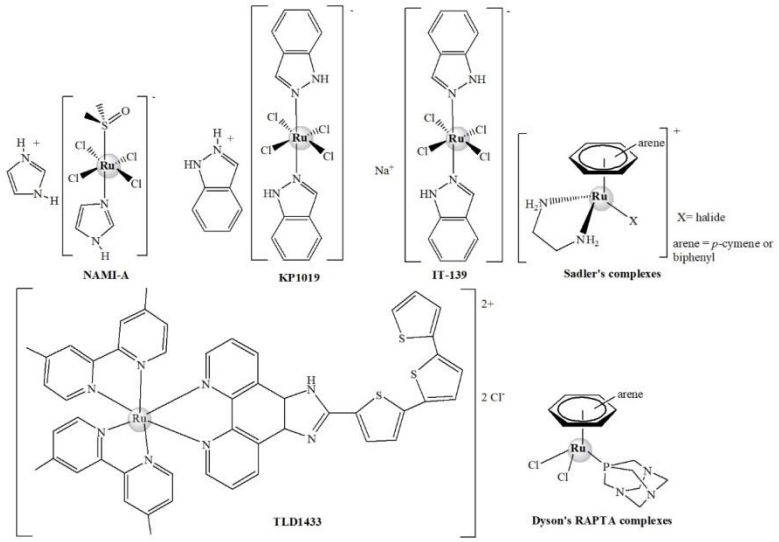

Figure 1. The most popular Ru(III) and Ru(II) complexes are prodrugs.

They also provide less toxicity than platinum-based complexes due in part to the skill of ruthenium complexes to mimic the behavior of iron and bind to biocompounds [17]. As a result of this entire development process, the half-sandwich ruthenium(II) complexes have been identified and studied as potential therapeutic agents [18,19]. Several factors can influence the cytotoxic activity of half-sandwich Ru(II) complexes. Cytotoxic activity is significantly affected by the choice and structure of ligands coordinated to the Ru(II) center. Different ligands can alter the complex's electronic and steric properties, thereby impacting their interactions with biological targets like DNA or proteins. The composition and substituents of the arene ligand can affect cytotoxicity by altering the complex's reactivity. The complex's overall geometry and steric hindrance can also influence its cytotoxic activity by affecting the accessibility of the complex to its target.

Additionally, the redox properties of the Ru(II)-half-sandwich complex may affect cytotoxicity through involvement in redox reactions or the generation of reactive oxygen species [20-22]. The anticancer activity of half-sandwich Ru(II) complexes is demonstrated through various mechanisms. These mechanisms consist of cellular uptake, activation, DNA binding, disruption of DNA structure, production of reactive oxygen species (ROS), and disruption of cellular processes. Cancer cells take up these complexes via different mechanisms and subsequently undergo biological activation within the cell. They were binding to DNA, resulting in structural disruptions and conformational alterations.

Moreover, they produce reactive oxygen species (ROS) in cancer cells. These complexes can disrupt cellular processes by interacting with proteins and enzymes. The Ru(II) complex and the type of cancer being treated determine the specific mechanisms of action.

Current research is focused on exploring and improving the anticancer properties of these complexes [8,23-25].

In contrast to the cyclometalated structural complexes of ruthenium, half-sandwich Ru(II) complexes are gaining increasing interest due to their high anticancer efficiency [26,27]. The common notation formula of half-sandwich Ru(II) complexes is defined [(η^6-Ph*)Ru(L^L)Z]X, where Ph* can be the electron-rich phenyl group and its derivatives, L^L is a different type of chelating ligand, while Z is the out-group and X is designated as the counterion. Such complexes (a three-legged piano stool structure) are octahedral, and the arene group occupies the three coordination zones. In structure, each moiety has a specific effect on anticancer activity, among which the most studied chelating ligands (L^L) [28]. The Ru(II) complexes with a "piano-stool" structure containing chloride and N-donor ligands generally have good aqueous solubility with sufficient lipophilicity required to cross the cell membrane. The arene-type ligands stabilize the oxidation state of ruthenium(II), making Ru(II) complexes more kinetically unstable compared to ruthenium(III) complexes. In addition, these complexes can provide hydrogen bonding and π-stacking in biological activity [29,30]. *N*, *N*-type ligands containing benzimidazole rings are remarkable for their biological activities, and the ligands in this study are good representatives of these derivatives. Benzimidazole's chirality, steric, and electronic aspects can be freely modified in such ligands. Although many efficacies of ruthenium complexes containing the biologically active and medically necessary benzimidazole ligand have been investigated [31,32], the availability of metal complexes with derivatives of the 2-(2′-quinolyl)benzimidazole-type ligands remains limited, including the synthesis and catalytic activity studies of the half-sandwich Ru(II) complexes with 2-(2′-quinolyl)benzimidazoles by our group [33-38]. Anticancer activity studies of especially 2-(2′-quinolyl)benzimidazoles-Ru(II) complexes will add a great innovation in this field.

The present study reports on the anticancer efficiencies of ruthenium (II) complexes containing 2-(2'-quinolyl)benzimidazole (**C1-C4**) on U373 GBM cells. In a previous study, our group investigated their transfer hydrogenation activity under open-air conditions [37,38]. Under the conditions studied, the synthesized complexes remain stable both in solution and in solid phase in the presence of humidity and oxygen. The molecular mechanisms of cancer cell death induced by metal-containing complexes are not very clear, and in vitro, anticancer mechanisms of half-sandwich ionic Ru(II) complexes based on benzimidazole-quinoline ligand and *p*-cymene group have been rarely reported. Hence, four ruthenium(II) complexes, **C1-C4**, with a 2-(2′-quinolyl)benzimidazole-based ligand prepared [38], and in vitro, cytotoxic and apoptotic properties of the complexes have been investigated. We hope our results will contribute to developing new chemotherapeutic agents.

2. Materials and Methods

2.1. Materials and reagents.

Dimethyl sulfoxide (DMSO, cas no.: 67-68-5) was obtained from Carlo Erba (Germany). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, cat no.: 475989-1GM) was purchased from Merck (Germany). Trypsin-EDTA (T4049-100ML), Dulbecco's Modified Eagle Medium (DMEM, D6429-500ML), and Paclitaxel (cas no.: 33069-62-4) were purchased from Sigma-Aldrich (Germany). The Fetal Bovine Serum (FBS, cat no.: FBS-HI-11A) and Penicillin/streptomycin (cat no.: PS-B)mix were obtained from Capricorn (Germany). All operations were performed outdoors unless otherwise stated. A series of

ligands (**L3-L5**, Figure 2) derived from 2-(2′-quinolyl)benzimidazole [39,40] (QuBim, **L1**) and 2-(2′-quinolyl)-5,6-dimethylbenzimidazole [41] (QuDmBim, **L2**) which are an *N*, *N*-type ligands have been synthesized, then the half-sandwich complexes (**C1-C4**) of Ru(II) with NN-type ligands (QuBim = **L1** and the substituted ligands **L3-L5** derived from QuBim and QuDmBim) have been prepared by cleavage of $[(\eta^6\text{-}p\text{-cymene})Ru(\mu\text{-}Cl)]_2$ dimer according to the published procedure [37,38] (Figure 2), as well as purification methods of the resulting compounds and characterization results with methods such as NMR, UV-Vis, FT-IR, elemental analysis, HRESI-MS/MS and X-ray diffraction are given in the paper published by our group [37,38]. In the mentioned publications, besides Ru(II)-catalyzed transfer hydrogenation (TH) of acetophenone to secondary alcohols of a series of half-sandwich Ru(II) complexes, including those in this study, the thermal and electrochemical properties of some selected complexes were examined.

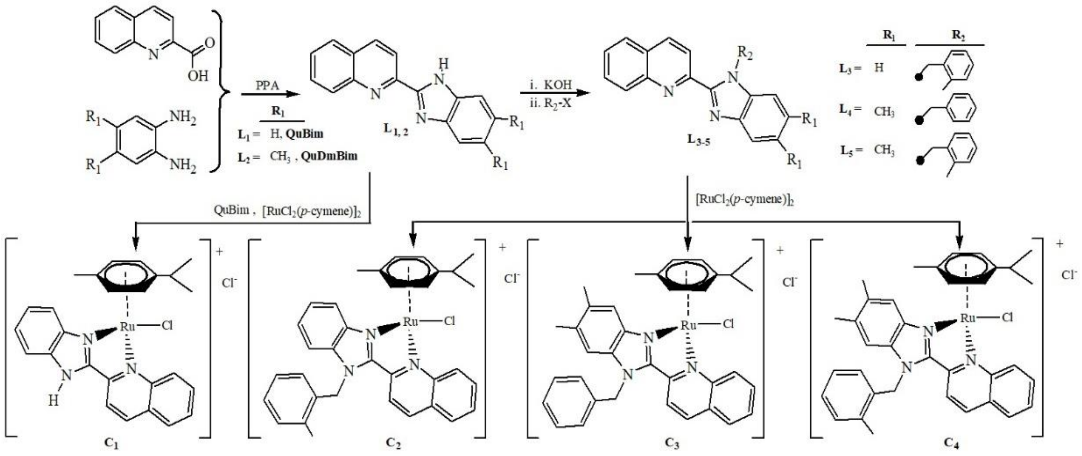

Figure 2. Synthesis of the quinoline-based ligands and Ru(II) complexes.

2.2. Cell culture and MTT assay.

In this study, we used human glioblastoma (U373) and embryonic kidney (HEK293) cell lines obtained from the European Collection of Cell Cultures (ECACC). The cells were grown in DMEM, supplemented with 10% FBS, and 1% penicillin/streptomycin mix. Cells were cultured in a 100 mm culture dish at 37°C and 5% CO_2. Cells were passaged every 2-3 days when confluency reached 70-80%. Cells were plated in a 96-well plate (Costar, Corning, USA) at a density $2x10^3$ and treated with various concentrations (7.8-125 µM) of compound dissolved in DMSO (not exceeding 0.5%). After 24h, 10 µL MTT reagent (5 mg/mL) was added to each well and incubated for 4h. The formazan dye was dissolved in 50 µL DMSO. The absorbance was measured at 590 nm with a microplate reader (Epoch, BioTek, USA). The MTT assay was performed as previously described [42]. The study was performed in triplicate, and cell viability was calculated as a percentage of the control. The obtained data were used to estimate the EC50 values. Calculations were performed using GraphPad Prism® software v.9 (San Diego, California USA).

2.3. Evaluation of apoptosis by Annexin V-APC/7-AAD staining.

The apoptosis was evaluated using the Annexin V-APC/7-AAD Apoptosis Kit according to the manufacturer's instructions (Elabscience, USA, cat no.: E-CK-A218) as previously described [43]. U373 cells were seeded in 6-well plates and treated with compounds, or they were incubated in a culture medium containing DMSO (v/v) (negative control) and 0.2

mM hydrogen peroxide (positive control). After 24h of incubation, cells were harvested with Trypsin-EDTA. U373 cells were collected, washed twice with PBS, and finally suspended in a cold Annexin binding buffer. Cells were stained with 5 µL of Annexin V-APC and 10 µL 7-AAD and incubated for 15 minutes in the dark. Finally, 400 µL of Annexin binding buffer was added to each sample and analyzed using CytoFLEX Flow Cytometer (Beckman Coulter, USA). CytExpert Software was used to calculate the percentage of viable, early apoptotic, late apoptotic, and necrotic cells.

2.4. Gene expression analysis by RT-qPCR.

Total RNA isolation was performed using the innuPREP RNA Mini Kit 2.0 (Analytik Jena, Germany), and the OneScript® Plus cDNA Synthesis Kit (ABM, USA) was used for cDNA synthesis according to the manufacturer's protocol. RT-qPCR was performed using ABM KiloGreen 2X qPCR MasterMix (USA) in an Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc., USA). The relative gene expression levels were normalized to GAPDH, and primer sequences for all genes were acquired from the NCBI database. For the determination of mRNA levels, the $2^{-\Delta\Delta Ct}$ method was used as described previously [44]. The data were analyzed using the GeneGlobe Data Analysis Center (Qiagen) tools. The sequences of primers are presented in Table 1.

Table 1. Sequences of primers used for RT-qPCR.

Gene	Sequences	Accession code
GAPDH	Forward: GTCTCCTCTGACTTCAACAGCG Reverse: ACCACCCTGTTGCTGTAGCCAA	NM_002046
Bcl-2	Forward: ATCGCCCTGTGGATGACTGAGT Reverse: GCCAGGAGAAATCAAACAGAGGC	NM_000633
Bax	Forward: GTCTCCTCTGACTTCAACAGCG Reverse: ACCACCCTGTTGCTGTAGCCAA	NM_004324
CASP3	Forward: GGAAGCGAATCAATGGACTCTGG Reverse: GCATCGACATCTGTACCAGACC	NM_004346
CASP8	Forward: AGAAGAGGGTCATCCTGGGAGA Reverse: TCAGGACTTCCTTCAAGGCTGC	NM_001080125
CASP9	Forward: GTTTGAGGACCTTCGACCAGCT Reverse: CAACGTACCAGGAGCCACTCTT	NM_001229

2.5. Statistical analysis.

GraphPad Prism 9.0 software was used for statistical analyses. The results were presented as the means±SD of at least three replicates. One-way ANOVA analyzed the statistical differences between the groups. P-values <0.05 were statistically significant.

3. Results and Discussion

3.1. Cytotoxicity against cancer and normal cell lines.

Cytotoxic effects of **C1-C4** were performed using MTT assay as described in the methods part. Cells were treated with different concentrations (7.8, 15.6, 31.25, 62.5, and 125 µM) of compounds for 24 h. All compounds significantly inhibited the viability of both U373 and HEK293 cells in a dose-dependent manner (Figure 3). The most inhibitory effects were observed in compounds **C1**, **C3**, and **C4,** which were about 90% in U373 cells after treatment with 31.25µM of these compounds. Similarly, C1, C3, and C4 cytotoxic effects were observed in non-cancerous HEK293 cells (80%) in the same concentration. The EC50 values were calculated as 7.49, 20.78, 3.73, and 2.09 µM for **C1**, **C2**, **C3**, and **C4**, respectively (Figure 3).

On the other hand, EC50 values were found to be 12.64, 30.15, 7.92, and 3.78, respectively, in the HEK293 cell line. These results show that all compounds can be effective for U373 cells at lower doses than non-cancerous HEK293 cells.

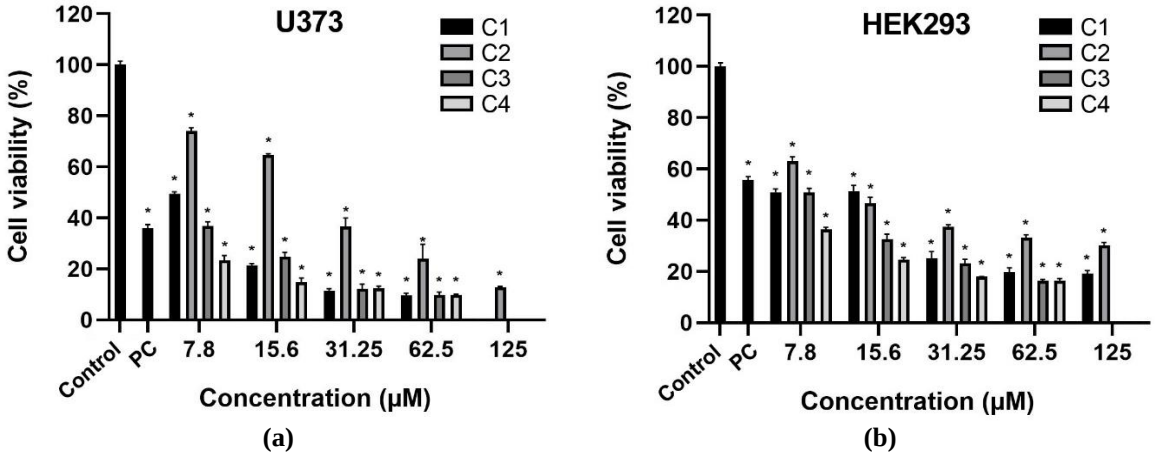

Figure 3. The cytotoxic effects of compounds against **(a)** U373; **(b)** HEK293 cells. PC: Positive control – paclitaxel (5 nM). Data are mean values of two independent replicates. *P<0.05 compared with the control group.

3.2. Flow cytometric analysis.

The type of cell death was evaluated in U373 cells using flow cytometry (CytoFLEX, Beckman Coulter). The results are shown in Figure 4. EC50 compound concentrations were tested individually (7.49, 20.78, 3.73, and 2.09 μM for C1, C2, C3, and C4, respectively) for 24 h and compared with hydrogen peroxide (0.2 mM). Results showed that **C1-C4** significantly increased the percentage of U373 cells in the early stage of apoptosis (26.30, 43.78, 23.80, and 17.35%, respectively) compared to untreated control cells. Significantly lower percentages of late apoptotic and necrotic cells were detected. All these results showed that our compounds caused cell death by inducing apoptosis.

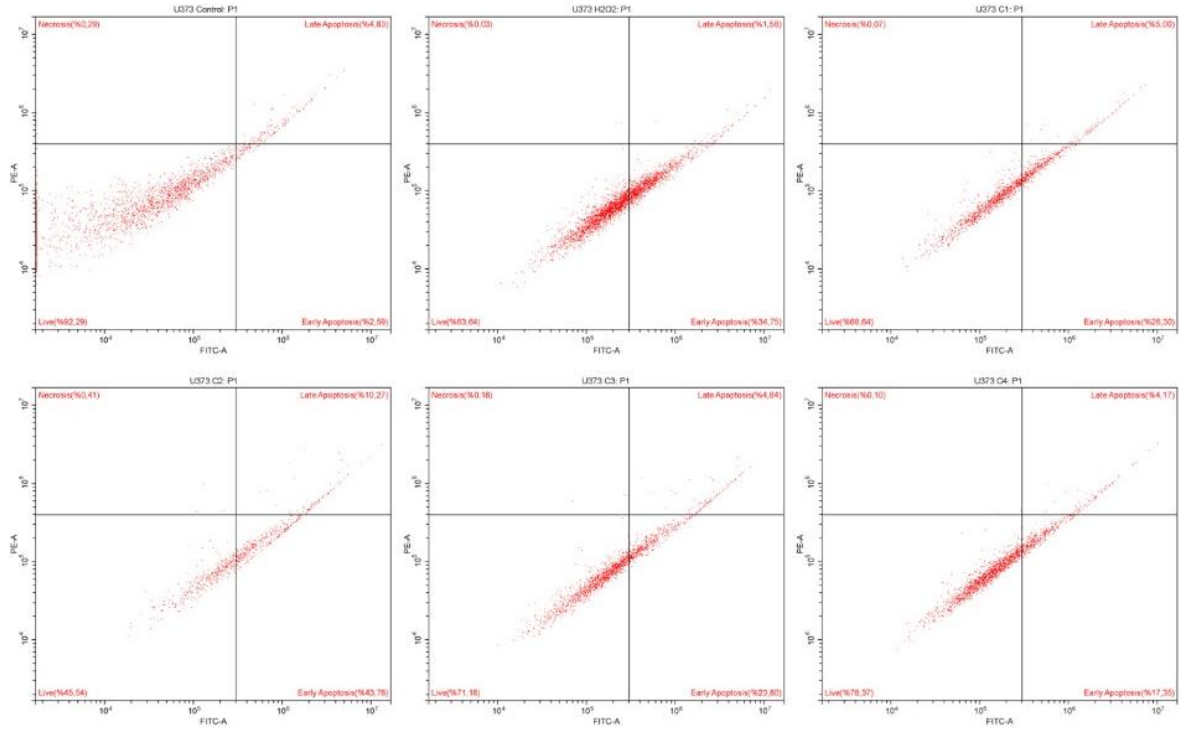

Figure 4. The apoptotic effect of the compounds against human glioblastoma (U373) cells by Annexin-V-APC assay using a flow cytometer.

3.3. Gene expression analysis.

To investigate the alteration of apoptosis pathways by compounds, a qPCR was used to find out changes in the expression levels of *Bax*, *Bcl-2*, *CASP3*, *CASP8*, and *CASP9* genes. After 24 h of incubation, *Bax* mRNA level was increased by 1.35-, 1.17-, 1.15-, and 1.79-fold in U373 cells (P<0.05) as a result of **C1**, **C2**, **C3**, and **C4** treatment, respectively. Similarly, *CASP3* mRNA level was increased 1.80-, 1.77-, 1.19-, and 2.27-fold. After 24 h, *CASP8* mRNA level was increased 1-41-, 1.71-, 1.59-, and 1.57- fold for **C1**, **C2**, **C3**, and **C4**, respectively. Besides, *CASP9* mRNA level was upregulated 2.18-, 2.03-, 1.88-, and 3.39-fold in the U373 cell line (P<0.05). However, **C1**, **C2**, **C3**, and **C4** treatment caused a 2.48-, 1.85-, 2.87-, and 1.35-fold decrease in *Bcl-2* mRNA levels compared to the control group (Figure 5).

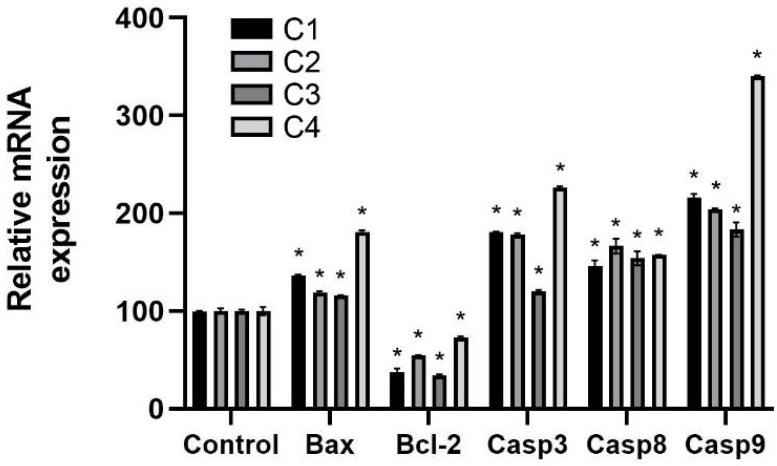

Figure 5. Effects of compounds on apoptosis-related gene expression levels in U373 cells. *P<0.05 compared with the control group.

Complexes resulting from the association of metals and heterocyclic ligands are of particular interest in bioinorganic chemistry because they form model compounds of metalloproteins. Inorganic drugs, which have become very popular in recent years, constitute an important part of modern medical drugs. For this reason, the chemical synthesis of *N*-coordinated metal-protein complexes has been extensively researched and studied in coordination chemistry [45]. Ruthenium is preferred because it shows very low cellular toxicity against healthy cells but is easily absorbed by tumor cells and quickly eliminated from the body [46]. Moreover, ruthenium is so effective because it exhibits properties similar to iron, has a good ligand exchange rate, and has a cytotoxic effect considerably lower than platinum [47].

In this regard, cytotoxic and apoptotic properties of previously synthesized and characterized four different half-sandwich ruthenium(II) complexes (C1-C4) with 2-(2′-quinoly)benzimidazole frameworks (L1-L5) were described for the first time in this study. As can be seen in Figure 3, all tested ruthenium complexes showed better cytotoxicity (lower EC50 values) for U373 than HEK293 cells. C3 and C4 had the highest cytotoxicity with an EC50 value of 3.73 and 2.09 μM for glioblastoma cells, respectively. When the relationship between structure and cytotoxic activity is examined, the structure that distinguishes the C1-C4 complexes from each other is R groups on the non-coordinating nitrogen and the 5,6-positions of benzimidazole (Figure 2). In the C1-C4 complexes, R groups on the *N* and 5,6-position, respectively, H and H (C1), 2-methylbenzyl and H (C2), benzyl and CH_3 (C3), 2-methylbenzyl and CH_3 (C4). While all complexes (C1-C4) showed strong activity against the human glioblastoma (U373) cancer cell line, it was seen that the C4 complex gave the best efficacy result. It is known that in Ru(II)(*p*-cymene) based complexes, it results from the better

hydrophobic interaction of the *p*-cymene part of the metal complexes with the cell membrane [48,49]. When we compare the cytotoxicity values of the complexes (C1-C4), the first point that draws attention is that the C2-C4 complexes are significantly higher than the C1 complex. This result showed us that the R groups on the non-coordinating nitrogen atom in the benzimidazole ring in the C2-C4 complexes contribute significantly to the activity, and the group bound to the benzimidazole structure has a significant role in the activity of the compound. The presence of methyl groups in benzylic aromatic on nitrogen and the benzimidazol-5,6-position in the C4 complex, it can be thought that the increase in the ruthenium-*N* bond strength as well as the increase in the metal electron density makes a significant contribution to the activity. It is known and interesting that the activity of antitumor ruthenium complexes is related to their ability to bind DNA [46,50]. It can be said that Ru metal, the *p*-cymene effect [44], and the benzimidazole skeleton [51] play an important role in the activity together. In a different study, which is an example of these effects, thiosemicarbazone ruthenium(II) complexes reduced cell proliferation in human ovarian cancer cells A2780 and the metastatic ovarian cancer cells OVCAR-3 in the dose range of 1-50 μM and high selectivity [24] and also ruthenium(II) tris-pyrazolylmethane complexes showed antiproliferative effects on different cancer cell lines [52]. Moreover, Ru(II) complexes containing *N,N*-donor different types of ligands showed cytotoxic effects on cancer cells that were consistent with the results of this study [27,53].

In addition to the effect of p-cymene and benzimidazole skeleton groups in the structure and the properties of R substituents on the nitrogen atom in benzimidazole skeleton (such as having a heteroatom, steric, electron withdrawer-donating, straight chain, aromaticity, chirality) on the anticancer activity, the redox potential between different oxidation steps of the ruthenium atom in the structure, shows a catalytic effect (especially in redox reactions) depending on the physiological environment. Biochemical changes in cancerization alter the physiological environment, allowing the ruthenium complex to activate selectively in cancerous cells [46]. It can be thought that the ruthenium complex binds to DNA due to the different ligand types in complex (C1-C4) and pseudo-octahedral geometry [37,38] in the Ru(II) complexes. As a matter of fact, it is known that ruthenium compounds differently affect the conformation of DNA than cis-platinum and its derivatives. It is also known that ruthenium compounds play a role in non-nuclear targets (such as mitochondria and cell surface) on the antineoplastic activity. for this reason, it offers lower toxicity than clinically used antitumor Pt(II) compounds, a new mechanism of action, potential to show no cross-resistance [50].

Our experiments showed that the cytotoxic activity of ruthenium complexes toward U373 cells can be attributed to the effective induction of apoptosis (Figure 4). Flow cytometry was used to evaluate types of cell deaths, including apoptosis. It is well established that it is a qualitative, quantitative, and accurate technique for analyzing apoptosis. Our result showed that ruthenium complexes drove apoptosis cell death in human glioblastoma U373 cells.

It is well-known that the two main apoptotic cell death pathways are extrinsic and intrinsic. Each pathway needs different caspase enzymes to activate it. Apoptosis triggers the intrinsic pathway by activating *CASP9* in response to cytochrome c release from mitochondria. As a result, this leads to the activation of effector caspases (3, 6, and 7) [54]. On the other hand, activation of *CASP8* that triggers *CASP3* activation is observed in the extrinsic pathway of apoptosis [55]. We detected Ru(II) complexes that increased the pro-apoptotic mRNA levels and decreased anti-apoptotic *Bcl-2* mRNA levels. Figure 5 showed that 24 h treatment with compounds caused significant increases in the mRNA expression levels of *CASP3* and *CASP9*

(apoptotic) genes and a significant decrease in *Bcl-2* (anti-apoptotic) gene expression. Treatment of C4 upregulated the expression of *Bax*, *CASP3*, and *CASP9*, which is related to apoptosis. C4 at a concentration of 2.09 μM increased the expression of *CASP9* (3.39-fold) more so than other complexes (Figure 5). Furthermore, treatment caused an increase in the *Bax/Bcl-2* ratio as well. Moreover, all of the synthesized compounds had increased mRNA levels of *CASP8* (Figure 5). All of these results showed that both intrinsic and extrinsic pathways of apoptosis were involved in cell death induced by these compounds. The apoptotic effects of Ruthenium complexes containing bis-benzimidazole derivatives were found in several distinct human cancer types, such as adenocarcinoma alveolar basal epithelial cells A549 [56], breast cancer cell MCF7 and colorectal cancer cells Caco-2 [57]. Li *et al*. [58] reported that treatment with bis-benzimidazole derivatives inhibited cell proliferation of the malign melanoma cells A375 and caused the induction of caspase-dependent apoptosis.

Moreover, the TUNEL-DAPI assay was used to evaluate caspase activation. Another study showed that the Ru(III) complex with 2-aminomethyl benzimidazole (AMBI) inhibits proliferation in MCF-7 and human colon carcinoma HCT-116 cells. Furthermore, it has been shown that the AMBI causes apoptosis in both cell lines by percent to 23.1 and 33.1% [59]. These results are consistent with the results of our study.

4. Conclusions

Herein, a series of the half-sandwich ruthenium(II) complexes framed by 2-(2′-quinoly)benzimidazole were found to have cytotoxic and apoptotic effects at low concentrations in glioblastoma cancer cell line, and they are more selective for these cells than for non-cancerous cell line. These complexes showed induction of apoptosis via activation of caspases with upregulation of Bax and downregulation of Bcl-2. All these results showed the potential use of ruthenium(II) complexes with 2-(2′-quinoly)benzimidazole frameworks as a new therapeutical strategy for glioblastoma. Further studies will be required to test this hypothesis.

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Conflicts of Interest

The authors declare no conflict of interest.

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