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Differences in the Cellular Response and Signaling Pathways between Cisplatin and Monodentate Organometallic Ru(II) Antitumor Complexes Containing a Terphenyl Ligand

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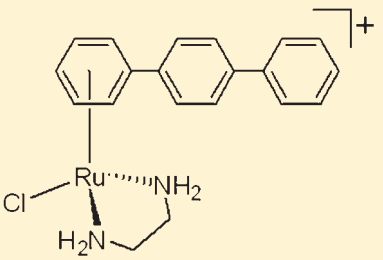
ABSTRACT: The new monofunctional Ru(II)-arene complex $[(\eta^6\text{-arene})\text{Ru(II)}(\text{en})\text{Cl}]^+$, where en = 1,2-diaminoethane and the arene is *para*-terphenyl (complex **1**) exhibits promising cytotoxic effects in human tumor cells including those resistant to conventional cisplatin (*J. Med. Chem.* **2008**, *51*, 5310). The present study is focused on the cellular pharmacology of **1** to elucidate more deeply the mechanisms underlying its antitumor effects. We have identified several cellular mechanisms induced by **1** in human ovarian carcinoma cells, including inhibition of DNA synthesis, overexpression and activation of p53, expression of proapoptotic proteins $p21^{WAF1}$ and Bax, G_0/G_1 arrest, and nuclear fragmentation as a result of apoptotic, and, to a much lower extent, also necrotic processes. Thus, **1** inhibits growth of the cancer cells through induction of apoptotic cell death and G_0/G_1 cell cycle arrest. Further investigations have shown that **1** induces apoptosis by regulating the expression of Bcl-2 family proteins. There were significant differences in cellular responses to the treatment with **1** and with conventional cisplatin, particularly in the kinetics and the extent of these responses. In addition, the distinct p53 activation profile of **1** compared with cisplatin provides an explanation for the activity of this ruthenium drug against cisplatin-resistant cells. Hence complex **1** may provide an alternative therapy in patients with acquired cisplatin resistance, particularly with respect to its very low mutagenicity and different mode of action compared to platinum antitumor drugs in clinical use.



KEYWORDS: ruthenium drug, antitumor, apoptosis, mutagenicity, p53 protein, cell cycle arrest

INTRODUCTION

Numerous ruthenium coordination complexes have been shown to exhibit promising anticancer activity. This fact prompted intense research focused on understanding the mechanism underlying biological effects of these metallodrugs. Several factors which might be involved in the mechanism underlying the cytotoxic effects of ruthenium complexes have been proposed, such as inhibition of metastases,[1] DNA damage,[2,3] production of reactive oxygen species,[4] interactions with proteins,[5−11] activation of genes of the endoplasmic reticulum stress pathway,[12] and triggering apoptosis.[5,13,14]

Organoruthenium complexes containing arene ligands received recently considerable interest since some of them have been shown to exhibit promising anticancer activity, including activity against cancer cells resistant to conventional cisplatin (*cis*-diamminedichloridoplatinum(II)).[15,16]

We reported recently a comparison of the three new isomeric complexes $[(\eta^6\text{-arene})\text{Ru(II)}(\text{en})\text{Cl}]^+$, where en =1,2-diaminoethane and the arene is *para*-, *ortho*-, or *meta*-terphenyl (complexes **1**, **2**, and **3**, respectively, Figure 1).[17,18] It was shown that **1** exhibits cytotoxic effects in human tumor cells including in tumor cells resistant to conventional cisplatin, whereas **2** and **3** are much less potent. The potency of **1** appeared to be enhanced by its dual-mode of DNA binding involving combined intercalative and monofunctional (coordination) binding modes similar to other monofunctional Ru(II)-arene complexes containing multiring arenes.[19,20] In contrast, less cytotoxic complexes **2** and **3** bind to DNA via only a monofunctional coordination mode to DNA bases. Moreover, our earlier results demonstrated that there is also a correlation between the ability of these Ru(II)-arene complexes to affect the conformation and thermodynamic stability of DNA and "downstream" cellular processing of these adducts on the one hand and cytotoxicity of these Ru(II)-arene complexes on the other.[17,18] All of these results along with those obtained with other cognate monofunctional Ru(II)-arene complexes[19,21−24] were interpreted to mean that monodentate Ru(II) arene complexes belong to a class of antitumor ruthenium compounds for which structure−activity relationships can be formulated on the basis of their DNA binding mode.

Several metal-based antitumor compounds including ruthenium complexes produce cell death through the induction of apoptosis and/or necrosis.[5,13,14,25] Thus, a goal of the present

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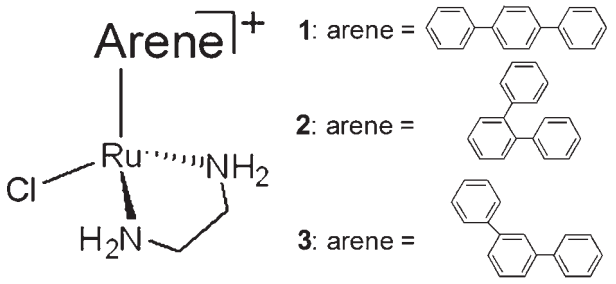

Figure 1. Structures of Ru(II) arene complexes. **1**, $[(\eta^6\text{-}p\text{-terp})\text{Ru(en)Cl}]^+$; **2**, $[(\eta^6\text{-}o\text{-terp})\text{Ru(en)Cl}]^+$; **3**, $[(\eta^6\text{-}m\text{-terp})\text{Ru(en)Cl}]^+$.

work was to find out whether the cytotoxic effect of the most effective complex of the class of monofunctional Ru(II)-arene compounds containing the multiring terphenyl ligand, complex **1**, is related to its capacity to induce apoptosis or necrosis and cell-cycle perturbations. In addition, the antitumor effects of metallodrugs may be associated with the presence of functional tumor suppressor protein p53.[26] In response to treatment of the cells with DNA damaging metallodrugs, DNA binding of p53 protein may be activated, and numerous response genes, such as those associated with promoting cell cycle arrest and apoptosis or with its inhibition, may be transactivated. Therefore, the objective of the present work was also to investigate the expression of proteins such as p53 and some closely related proteins, following treatment of tumor cells with **1** and to compare these results with the effects of conventional cisplatin. In addition, an important issue with respect to the clinical use of antitumor transition metal-based drugs is whether these are mutagenic. We therefore assessed the mutagenicity of **1** using the *in vitro* mammalian cell gene mutation test[27] and compared it with that of less effective isomeric Ru(II)-terphenyl complexes **2** and **3** and with clinically used cisplatin.

MATERIALS AND METHODS

Chemicals. Complexes **1**, **2**, and **3** were prepared by the methods described in detail previously.[17] Cisplatin, dimethyl sulfoxide (DMSO), and 6-thioguanine (6-TG) were obtained from Sigma-Aldrich s.r.o., Prague, Czech Republic. The stock solutions of Ru(II)-arene complexes and cisplatin were freshly prepared before use in DMSO (Sigma-Aldrich s.r.o., Prague, Czech Republic). The final concentration of DMSO in cell culture medium did not exceed 0.25% (v/v). Acrylamide and bis(acrylamide) were purchased from Merck KgaA (Darmstadt, Germany), sodium dodecyl sulfate (SDS) from Serva (Heidelberg, Germany), and ^{3}H-thymidine from MP Biomedicals LLC (Irvine, CA).

Cell Lines. The A2780 and CH1 human ovarian carcinoma cell line (cisplatin sensitive) was kindly supplied by B. Keppler (University of Vienna, Austria). A2780 cells were grown in RPMI 1640 medium (GIBCO, Carlsbad, CA) supplemented with gentamycin (50 μg mL^{-1}; Serva, Heidelberg, Germany) and 10% heat inactivated fetal bovine serum (GIBCO, Carlsbad, CA). V79 Chinese hamster lung fibroblast cells were obtained from Dr. M. Piršel (Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia). V79 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamycin and 10% heat-inactivated fetal bovine serum (Carlsbad, CA). The cells were cultured in a humidified incubator at 37 °C in a 5% CO_2 atmosphere and subcultured 2−3 times a week at an appropriate plating density.

Cytotoxicity. The tumor cell line A2780 was cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, gentamycin (50 μg mL^{-1}) at 37 °C in an atmosphere of 95% of air and 5% CO_2. The metallodrugs were added to final concentrations from 0 to 256 μM. Cell death was evaluated after 72-h treatment, or the cells were exposed to the complex for 24 h; then, after removal of the complex, a fresh medium was added, and the cells were incubated for 48 h of recovery time. Cell death was evaluated by using a system based on the tetrazolium compound MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] in the same way as described recently.[17,18] IC_{50} or IC_{75} values (compound concentrations that produce 50% or 75%, respectively, of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in quadruplicate.

Inhibition of DNA Synthesis. The effect of Ru(II)-arene complexes on DNA synthesis was determined using the [^{3}H]-thymidine incorporation assay. A2780 cells were seeded at density of 30 000 cells/cm^2 and incubated overnight. Then the ruthenium compounds or cisplatin were added at the indicated concentrations for 1, 2, 6, and 24 h. After the incubation with the compounds, fresh medium containing 2 μCi mL^{-1} [^{3}H]-thymidine was added to the cells. Following an incubation for 30 min at 37 °C, the cells were washed with ice-cold PBS (137 mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl) and harvested. The cells were lysed using 1 M NaOH and washed with ice-cold 5% trichloroacetic acid for 20 min followed by 95% ethanol. Radioactivity incorporated into DNA was determined using a scintillation spectrometer.

Detection of Apoptosis and Necrosis. The cell death detection ELISA plus kit (Roche Molecular Biochemicals, Mannheim, Germany) was used as an indicator of apoptosis and necrosis.[28] In this assay, internucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes. Briefly, after centrifugation (200 *g*), 20 μL of the supernatant was used in the enzyme-linked immunosorbent assay (ELISA) for detection of necrosis. A2780 and CH1 cells were resuspended in 200 μL of the lysis buffer supplied by the manufacturer and incubated for 30 min at the room temperature. After pelleting of the nuclei (200 g, 10 min), 20 μL of the supernatant (cytoplasmic fraction) was used in the ELISA for detection of apoptosis following the manufacturer's standard protocol. Following incubation with peroxidase substrate for 20 min, absorbance was determined at 405−490 nm (reference wavelength) with a microplate reader (absorbance reader Sunrice Tecan Schoeller). Signals from wells containing the substrate only were subtracted as background. Other details of this assay and data analysis were performed according to the manufacturer's instructions.

Western Blotting. A2780 cells were incubated at 37 °C in a 5% CO_2 atmosphere with cisplatin and complex **1** for 24 or 48 h. At each concentration and time point, the treated cells were harvested, washed twice in cold PBS, and lysed in lysis buffer containing 150 mM NaCl, 1% Nonidet NP-40, and 50 mM Tris-HCl/5 mM EDTA (pH 8.0) supplemented with 1 mM PMSF, protease, and phosphatase inhibitor cocktails (Roche, s.r.o., Praha, Czech Republic). During lysis, cells were incubated at 4 °C and continuously vortexed. After lysis, the cell lysates were

centrifuged at 14 000 *g*, 4 °C for 30 min. The protein concentration of the supernatant was determined by the Bradford assay using bovine serum albumin (BSA) as a standard. The lysate containing protein (10 μg) was subjected to SDS-polyacrylamide (PAA) gel electrophoresis on a 10% PAA gel in loading buffer (50 mM Tris, 2% SDS, 0.1% bromphenol blue, 10% glycerol) and blotted onto nitrocellulose membrane (Bio-Rad, spol. s.r.o., Praha, Czech Republic) in a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Detection of p53 was carried out by overnight incubation of the membrane with mouse monoclonal anti-p53 (#sc-263, 1:200, Santa Cruz Biotechnology, Inc., CA) primary antibody. The membrane was then washed with TBS-Tween and incubated with secondary horseradish peroxidase conjugated antimouse antibody (IgG-HRP, Thermo Scientific Pierce Antibodies, GeneTICA, s.r.o., Prague, Czech Republic) (1:20 000 dilution) for 2 h. Equal loading of transferred proteins was verified by detection of β-actin using mouse monoclonal anti-β-actin (A5441, 1:10 000, Sigma-Aldrich Corp., Prague, Czech Republic) primary antibody, followed by incubation of the membrane with secondary horseradish peroxidase conjugated antimouse antibody (IgG-HRP) (1:20 000 dilution) as detailed above. Visualization of proteins was performed using Super Signal West Pico Chemiluminiscent Substrate Kits (Thermo Scientific Pierce Antibodies, GeneTICA, s.r.o., Prague, Czech Republic). Quantification of visualized bands was performed by densitometry using AIDA software (Advanced Image Data Analyzer). Western blotting of proteins Bax, Bcl-2, and p21 was made as described above (for p53 protein) with some modifications: SDS-PAA gel electrophoresis was made on a 15% PAA gel. The following primary antibodies were used for detection of these proteins: anti-Bcl-2 (#sc-65392, 1:200, Santa Cruz Biotechnology, Inc., CA), anti-p21 (#sc-817, 1:200, Santa Cruz, CA), and anti-Bax (4H32, 1:200, Santa Cruz Biotechnology, Inc., CA) (#sc-70407).

Cell Cycle Analysis. At each time point, floating cells were collected, and attached cells were harvested by trypsinization. Total cells (floating + attached) were washed twice in PBS (4 °C), fixed in ethanol, and stored at −20 °C. After pelleting, the cells were stained with staining solution [propidium iodide (20 μg mL^{-1}) in PBS + ribonuclease A (DNA free, 5 U mL^{-1})] for 30 min at 37 °C in the dark. The DNA content of the cells was analyzed using flow cytometry (Cell Lab Quanta SC, Beckman Coulter). The analysis of cell cycle distribution was then performed by using MultiCycle AV software (Phoenix Flow System).

Colony Forming Ability of V79 Cells and Determination of 6-Thioguanine Mutations by HPRT/V79 Assay. Mutation frequency was detected by counting 6-TG resistant colonies in V79 cells in the same way as described previously.[29,30] Briefly, V79 cells were seeded (3×10^5, in triplicate per sample) in Petri dishes (100 mm) and incubated at 37 °C in a 5% CO_2 atmosphere. On the third day the cells were exposed to cisplatin and complexes **1**−**3** at the concentration of 5×10^{-6} M for 2 h. The HPRT/V79 mutation assay was used for detection of 6-TG resistant mutations. After the treatment, the cells were trypsinized and plated (3.5×10^5, in triplicate per sample) on Petri dishes (100 mm) for further cultivation.

For colony-forming ability (CFA) the cells were plated on Petri dishes (3×10^2, 60 mm, in triplicate per sample). On the seventh day after treatment, the cells were stained with methylene blue (1% solution), and the number of colonies was counted. From the ratio of the number of colonies/cells plated, the percentage of CFA was calculated.

Table 1. IC_{50} Mean Values (μM) Obtained for Complex 1 and Cisplatin[a]

complex	A2780	CH1
1 (24 h)[b]	21 ± 1	9.2 ± 0.9
cisplatin (24 h)[b]	4.2 ± 0.8	3.8 ± 0.7
1 (72 h)[c]	4.0 ± 0.9	2.2 ± 0.3
cisplatin (72 h)[c]	2.8 ± 0.7	0.9 ± 0.1

[a] The experiments were performed in quadruplicate. [b] The drug treatment period was 24 h. [c] The drug treatment period was 72 h.

The cells for further cultivation were kept by regular subculture at a certain cell density to avoid overcrowding. Each sample was processed as follows: the cells were plated (a) on five Petri dishes (10 mm) at a density of 2×10^5 cells per dish for detection of 6-TG; after the cells were attached, the selective agent 6-TG was added in the final concentration of 5 μg mL^{-1}; (b) on the three Petri dishes (60 mm) at a density of 3×10^2 cells per dish for estimation of the viability of the cells; and (c) on three Petri dishes (100 mm) at a density of 3.5×10^5 cells per dish for further cultivation. On the seventh day of expression, the yield of 6-TG mutations was measured. Colonies of mutations were stained by methylene blue (1% solution) and counted 10 days after adding 6-TG. The frequency of 6-TG mutations per 10^5 viable cells was calculated on the seventh day of sampling.

Statistical Analysis. Statistical evaluation of the untreated control cells and drug treated cells was carried out using the Student's *t* test. If not stated otherwise a probability of 0.05 or less was deemed statistically significant.

RESULTS

Among the *ortho-*, *meta-*, and *para-* structural isomers of the monofunctional Ru(II)-arene complexes containing the multiring terphenyl arene ligand, the *para* isomer **1** exhibited the highest toxic effects in cancer cell lines, whereas **2** and **3** were much less cytotoxic.[17] Thus, **1** was selected for further more detailed studies focused on its cellular pharmacology. In addition, the structure and DNA binding profile of **1** is different from antitumor metallodrugs commonly used clinically, such as cisplatin and its analogues. Notably **1** shows higher potency toward cisplatin-resistant tumor cells compared to cisplatin sensitive cells, which suggests a mechanism of cytotoxicity for **1** different from that of cisplatin.[17,21] Therefore, experiments focused on the cellular pharmacology of **1** were also compared with those of conventional cisplatin.

Cytotoxicity. The cytotoxic activity of **1** and cisplatin was determined against cisplatin sensitive human ovarian carcinoma A2780 and CH1 cell lines. The tumor cell lines were incubated for 24 or 72 h with **1** or cisplatin, and the cell survival in the culture treated with metal complexes was evaluated as described in the Materials and Methods section. All complexes show activity, and their corresponding IC_{50} values are reported in Table 1. In general, the activity of **1** was lower than that of cisplatin, although the activity of **1** determined after 72 h treatment was almost comparable to cisplatin also in accordance with previously published data.[17]

Cell Death Detection. To analyze the characteristics of the cell death induced by **1** and to identify whether cell death is related to apoptotic or necrotic processes, the level of apoptosis and necrosis induced by this drug and cisplatin (at various concentrations) over 24 h was quantified by a specific ELISA

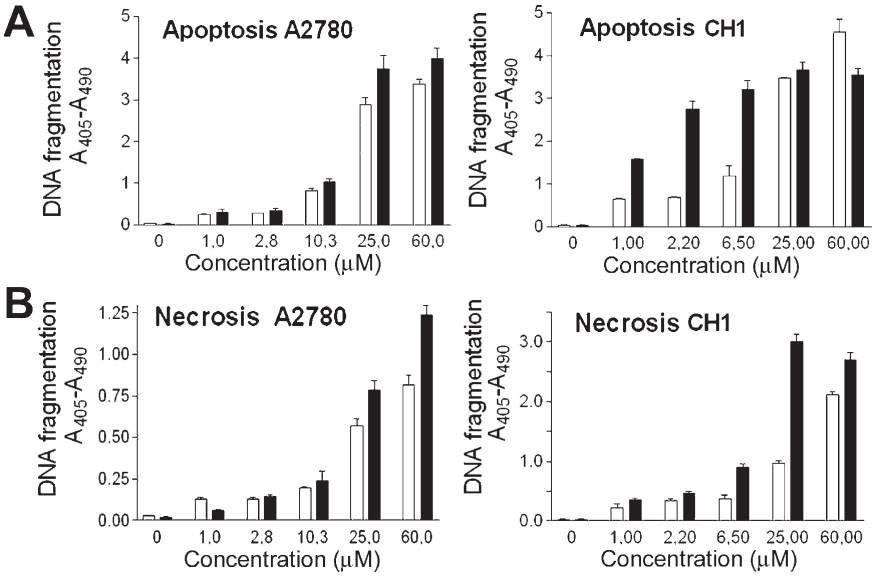

Figure 2. Effects of cisplatin (black bars) and complex **1** (white bars) on activation of apoptotic pathway (A) and necrosis (B) in A2780 (left panels) and CH1 (right panels) cells determined by DNA fragmentation ELISA assays. Cells were treated for 24 h with cisplatin or complex **1** at various concentrations. The results are expressed as mean of two independent experiments with duplicate runs.

kit. This analysis allows the appearance and relative amounts of cytoplasmic histone associated-DNA fragments (mono- and oligonucleosomes) to be measured after the induction of apoptosis or when these fragments are released from necrotic cells.

Figure 2A shows DNA fragmentation induced by both **1** and cisplatin in A2780 (left panel) and CH1 cells (right panel) as a result of apoptotic processes. These results demonstrate that the treatment with both antitumor agents led to concentration-dependent apoptotic events in both cell lines. Importantly, **1** induced a lower level of DNA fragmentation due to apoptosis in comparison with cisplatin in A2780 cells over the whole range of metallodrug concentrations used in these experiments. In CH1 cells, **1** was less effective in inducing DNA fragmentation at low concentrations compared to cisplatin, whereas **1** at high concentrations was at least as effective as cisplatin. A similar procedure was also used to detect the extent of necrosis induced by **1** or cisplatin. Importantly, the level of necrosis was significantly lower compared to apoptosis triggered by both metallodrugs and in both A2780 and CH1 cell lines, although the CH1 cells were more susceptible to necrosis compared to A2780 cells (Figure 2B).

Cell Cycle Analysis. The status of the cell cycle for cells treated with **1** and for comparative purposes also with cisplatin was analyzed. The analysis of cell cycle perturbation was performed using A2780 cells exposed to **1** or cisplatin for 24 h (the concentrations of the drugs were 4.0 and 25.0 μM) and for 72 h (the concentration of the drugs was 4.0 μM). An evaluation of the effects of **1** compared to untreated control A2780 cells showed several significant differences in cell cycle modulation, mainly at the higher concentration of the drugs after 24 h of treatment or after the 72 h treatment (at the lower concentrations of the drug; Figure 3). Exposure of A2780 cells to **1** or cisplatin caused the appearance of a population in the sub-G1 region of the profile, where apoptotic cells are found. Appearance of a subG$_1$ peak is consistent with the onset of internucleosomal DNA cleavage in late apoptosis.[31] Also, importantly, the treatment with **1** increased the G$_0$/G$_1$ populations, while peaks

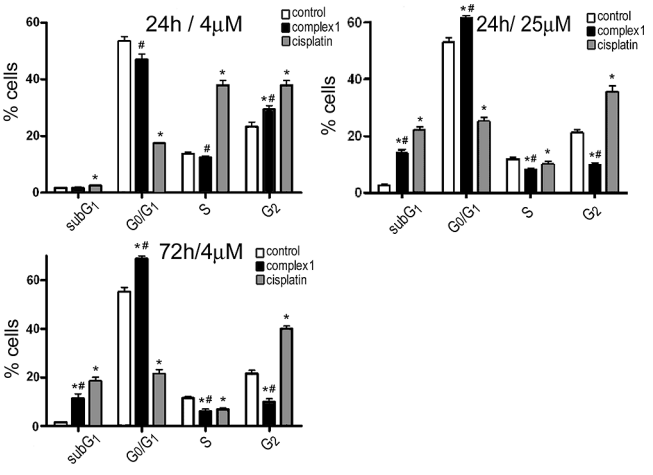

Figure 3. Effects of complex **1** or cisplatin on cell cycle distribution. Untreated (control) A2780 cells or A2780 cells treated for 24 or 72 h were harvested, fixed, stained with propidium iodide, and assessed for cell cycle distribution by FACS analysis. The estimated percentages of A2780 cells in different phases of the cell cycle are indicated. The results are expressed as mean ± standard deviations of three independent experiments with duplicate runs. The symbol (*) denotes significant difference ($p < 0.05$) from untreated control; the symbol (#) denotes significant difference ($p < 0.05$) between **1** and cisplatin.

corresponding to S and G$_2$ phases decreased (Figure 3). These results can be interpreted to mean that **1** induced the growth arrest in G$_1$ phase.

Different effects were observed for cisplatin, which produced more pronounced modifications of cell cycle already after a 24 h treatment at the concentration of the drug of 4 μM (Figure 3). There was a significant arrest of S phase and a shift of cisplatin-treated cells into G$_2$ phase. After a longer incubation period (72 h), cisplatin caused an accumulation in the G$_2$ phase. The fact that **1** and cisplatin had different effects on cell cycle progression suggests that the two drugs do not have the same mechanism of action.

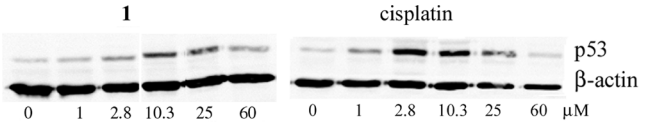

Figure 4. Western blot analyses of p53 protein levels in A2780 cells. The cells were not exposed (untreated control) or exposed to complex **1** (left panel) or cisplatin (right panel) at various concentrations (1−60 μM) as indicated and harvested after 24 h of sustained drug treatment. One representative experiment of three is presented. Equal loading is documented by detection of β-actin.

Table 2. Levels of Expression of p53 Protein after Treatment of A2780 Cells with Various Doses of Complex 1 or Cisplatin[a]

	0 μM	1 μM	2.8 μM	10.3 μM	25 μM	60 μM
1	1	2.1 ± 0.2	3.25 ± 0.01[e]	5.9 ± 0.8[d]	5.3 ± 0.1[e]	3.3 ± 0.1[e]
cisplatin	1	2.3 ± 0.2[b]	5.3 ± 0.7[c]	7.8 ± 1.4[c]	2.7 ± 0.2[b]	1.1 ± 0.1

[a] The cells were treated with the metal complexes at the concentrations in the range of 0−60 μM for 24 h. The data represent the mean ± standard deviations of three independent experiments. [b] Significantly different from control, $p < 0.1$. [c] Significantly different from control, $p < 0.01$. [d] Significantly different from cisplatin, $p < 0.5$. [e] Significantly different from cisplatin, $p < 0.05$.

Expression of p53 Protein. Since we demonstrated that **1** induced cell growth arrest and apoptosis, we also examined some aspects of the cellular mechanisms that may account for these processes. The activity of the tumor suppressor protein p53 has been reported to be induced by several anticancer drugs including cisplatin and its analogues,[26,32] p53 is stabilized, and its DNA binding is activated.[33] Therefore, the expression of the p53 protein was examined by Western blotting to compare with the pathway leading to the induction of A2780 cell death in response to DNA damage by **1**. We compared the effects of **1** with that in cisplatin using human ovarian A2780 cells, which express wild type p53.

In comparison with the untreated control, a concentration-dependent increase in p53 levels was observed for the treatment with lower concentrations of **1** or cisplatin for 24 h (Figure 4 and Table 2). The treatment with cisplatin was more efficient in the expression of p53 protein than that with **1**. In contrast, treatment with higher concentrations (>10.3 μM) led to a reduction of p53. The reduced level of p53 was not due to a decrease of cell density, as actin protein level remained unchanged under these conditions. It is reasonable to suggest that higher levels of the metallodrug may cause enhanced p53 degradation.[26,34] Thus, treatment of the A2780 cells with **1** resulted in significant and concentration-dependent changes in p53 expression. However, when this effect was compared with that induced by cisplatin, it was less pronouncedly manifested. Hence, although the accumulation of p53 proteins by the two drugs followed the same trend, there was a clear difference in intensities of this event.

Expression of Bcl-2, Bax, and p21 Proteins. The results described above showed that expression of p53 was significantly increased in response to the treatment of A2780 cells with **1**. Since p53 protein stimulates the transcription of several genes involved in cell growth arrest ($p21^{WAF1}$) or apoptosis (Bcl-2 family proteins, such as proapoptotic Bax and antiapoptotic Bcl-2), the effect of **1** on expression of these p53 target genes was studied in the A2780 cell line treated with the metal complex for

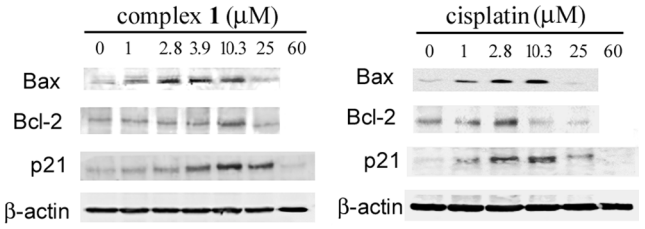

Figure 5. Western blot analyses of levels of Bax, $p21^{WAF1}$, and Bcl-2 proteins in A2780 cells. The cells were not exposed (untreated control) or exposed to complex **1** (left panel) or cisplatin (right panel) at various concentrations as indicated (1−60 μM) and harvested at 24 h of sustained drug treatment. One representative experiment of three is presented. Equal loading is documented by detection of β-actin.

24 h by Western blotting. Resulting immunoblots (Figure 5) and their evaluations (Table 3) demonstrate that the treatment with **1** induced accumulation of the cell cycle inhibitor $p21^{WAF1}$ as well as Bax and Bcl-2 proteins. The level of expression of these proteins was concentration-dependent and increased with increasing concentration of **1** (up to 10.3 μM). Interestingly, after treatment with higher concentrations of **1**, the amount of $p21^{WAF1}$, Bax, and Bcl-2 proteins started to decrease. The reduced level of $p21^{WAF1}$, Bax, and Bcl-2 was not due to a decrease of cell density, as actin protein level remained unchanged under these conditions. Hence, the proteins $p21^{WAF1}$, Bax, and Bcl-2 were degraded if the cells were treated with a high concentration of **1**. Importantly, the effect of **1** on the regulation of expression of proteins $p21^{WAF1}$ and Bax is much more pronounced compared to that of Bcl-2 protein; 10.3 μM complex **1** induced 22 and 6 times higher expression of p21 and Bax proteins, respectively, as compared to untreated cells, whereas the level of expression of Bcl-2 was increased only by a factor of 1.8.

The overall effect of **1** on the expression of $p21^{WAF1}$ and Bax proteins is qualitatively similar to that induced by cisplatin; that is, both metallodrugs stimulate expression of these proteins. According to previously described results,[35] p21 and Bax are overexpressed as a result of cisplatin treatment. However the levels of accumulation of these two proteins are different from those induced by the Ru(II)-arene complex **1** (Table 3) at the same concentrations of these drugs. In addition, consistent with literature data,[36] we found that the expression of Bcl-2 in A2780 cells was not significantly changed by 1−2.8 μM cisplatin and that it was down-regulated after treatment with 10.3 and 25 μM cisplatin. In contrast to this result, treatment with **1** led to a small, but significant ($p < 0.5$) increase in the expression of antiapoptotic Bcl-2 protein when the cells were treated with 1−10.3 μM complex **1**; when the concentration of **1** was further increased to 25 μM, the level of Bcl-2 expression decreased (Table 3). In aggregate, the correlation between the increase in p53 proteins and the activation of their target genes ($p21^{WAF1}$ and Bax) suggests that Ru(II)-arene complex **1** activates p53 protein functions similarly to cisplatin,[33] but with quantitatively different effects.

Inhibition of DNA Synthesis. In an attempt to elucidate the key events responsible for the observed reduction in cellular viability in cancer cell lines, the effects of complex **1** (exhibiting the highest potency) and complex **3** (with the lowest potency) on DNA synthesis were determined using [^{3}H]-thymidine incorporation assay. The A2780 cells were treated with **1** or **3**

Table 3. Levels of Expression of p21, Bax, and Bcl-2 Proteins after Treatment of A2780 Cells with Various Doses of Complex 1 or Cisplatin[a]

		0 μM	1 μM	2.8 μM	3.9 μM	10.3 μM	25 μM	60 μM
1	p21	1	2.0 ± 0.3^c	2.4 ± 0.3^c	15.8 ± 1.3^d	22.0 ± 2.8^d	13.1 ± 1.7^d	1.5 ± 0.4^b
	Bcl-2	1	1.2 ± 0.1^b	1.2 ± 0.3^b	1.33 ± 0.05^b	1.83 ± 0.05^b	0.7 ± 0.1^b	ND
	Bax	1	3.2 ± 0.4^c	3.9 ± 0.8^d	5.4 ± 0.5^d	6.0 ± 0.3^d	0.4 ± 0.1^b	ND
cisplatin	p21	1	3.4 ± 0.5^c	10.1 ± 1.7^c	ND	9.4 ± 2.4^c	2.9 ± 0.3^c	0.4 ± 0.1^b
	Bcl-2	1	0.93 ± 0.06^b	1.2 ± 0.2^b	ND	0.38 ± 0.02^c	0.25 ± 0.03^c	ND
	Bax	1	23.6 ± 4.4^c	19.7 ± 3.2^c	ND	8.8 ± 1.9^c	1.9 ± 0.2^c	ND

[a] The cells were treated with the metal complexes at the concentrations in the range of 0−60 μM for 24 h. The data represent the mean ± standard deviations of three independent experiments. [b] Significantly different from control, $p < 0.5$. [c] Significantly different from control, $p < 0.05$. [d] Significantly different from control, $p < 0.005$.

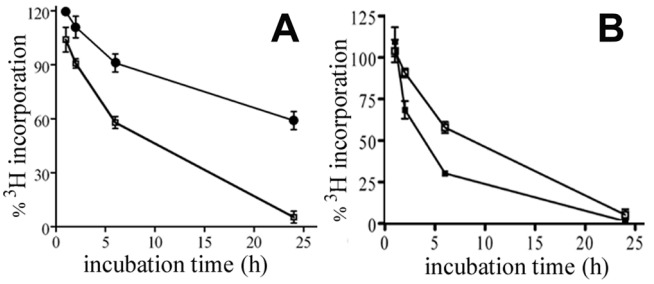

Figure 6. Effects of complexes **1**, **3**, and cisplatin on DNA synthesis in A2780 cells using [^{3}H]-thymidine incorporation assay. (A) Effects of **1** (□) and **3** (●) following continuous exposure for 1−24 h with equimolar concentrations (7.8 μM) and (B) **1** (□) and cisplatin (■) following continuous exposure for 1−24 h with equitoxic concentration of the compounds (7.8 μM complex **1** and 6.0 μM cisplatin; these values correspond to the IC_{75} values of **1** and cisplatin for A2780 cells treated with the metallodrugs for 72 h). The experimental points represent mean ± standard deviations of three independent experiments.

at equimolar concentrations (7.8 μM). This assay was carried out following a 1, 2, 6, and 24 h drug treatment period. The results suggest that both **1** and **3** caused a time-dependent decrease in DNA synthesis (Figure 6A). Furthermore, **1** appeared to be more potent in inhibiting DNA synthesis in this neoplastic-derived cell line than **3**, a trend which was consistent with the cytotoxicity data previously obtained using the MTT assay.[17] To compare DNA synthesis, the inhibitory effect of the most cytotoxic Ru(II)-arene complex **1** was compared with the effect of conventional cisplatin. These experiments were performed using equitoxic concentrations (IC_{75}) of cisplatin and **1** determined after 72 h of the incubation (6 and 7.8 μM, respectively). The results (Figure 6B) reveal that cisplatin is more effective in inhibiting [^{3}H]-thymidine incorporation compared to **1** at concentrations causing the same cytotoxic effects at shorter treatment times (2 and 6 h). Thus, although the inhibition of DNA synthesis by cisplatin and **1** at their equitoxic doses is equivalent in intensity after 24 h of incubation, there was a significant difference in the kinetics of these inhibition effects.

Colony Forming Ability of V79 Cells and Determination of 6-Thioguanine Mutations by HPRT/V79 Assay. HPRT, a forward-mutation assay which is appropriate for use in the initial assessment of the genotoxicity of test agents,[27] was used to evaluate the potential of Ru(II)-arene compounds to induce mutations at the *hprt* locus of V79 cells. V79 cells were exposed to 5 μM Ru(II) compounds and cisplatin for 2 h and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. The treated cultures were kept by regular subculture for 7 days, and mutant frequency was determined in medium containing the selective agent (6-TG) to detect mutant cells and in medium without 6-TG to determine the cloning efficiency (viability). Treatment of V79 cells with cisplatin and ruthenium complexes **1** and **3** resulted in cell lethality (23−27% of cell death, Table 4). In contrast, toxicity of complex **2** in V79 cells was not pronounced.

Table 4. Mutant Frequency of Cisplatin and Ruthenium Arene Complexes in V79 Cells[a]

	expression time: 0 min		expression time: day 7	
	cloning efficiency (%)	R^b	ML[c]	MF[d]
control	51 ± 4	1	2.2 ± 0.6	1
cisplatin	37.4 ± 3.6	0.73	8.7 ± 0.4	3.5[e]
1	38 ± 7	0.75	3.6 ± 0.7	1.3
2	53 ± 2	1.03	4.2 ± 1.1	1.8[f]
3	39 ± 3	0.77	4.9 ± 0.1	2.1[f]

[a] The cells were exposed to 5 μM Ru(II)-arene compounds **1**−**3** or cisplatin for 2 h. [b] Ratio of cloning efficiency in treated to untreated cells. [c] The number of 6-TG resistant mutations per 10^5 viable cells treated with complexes **1**−**3** or cisplatin; ML in control = spontaneous mutations. [d] Mutation frequency, MF = ML treated cells/ML control. [e] Significantly different from control, $p < 0.01$. [f] Significantly different from control, $p < 0.1$.

Table 4 also shows the results of HPRT mutation assay. Cisplatin, in accordance with previously published data,[37] produced a significant increase in mutation factor (MF). Interestingly, all ruthenium complexes tested in this work were significantly less mutagenic than cisplatin at equimolar doses. Importantly, complex **1**, the most cytotoxic agent of the three isomeric Ru(II)-terphenyl complexes against cancer cell lines,[17] showed the lowest mutagenic effect, not significantly different from the control.

■ DISCUSSION

Current platinum-based anticancer therapies are very successful in the clinic, but the discovery of new more efficient anticancer drugs with less toxicity and less sensitivity to resistance mechanisms remains a fundamental challenge. Active research in developing new therapies has led to extensive investigations into novel complexes based on an alternative transition metal. Ruthenium-based compounds have been recently explored for their cytotoxic effects. In addition, some of them have a surprisingly low general

toxicity and show activity even in cisplatin-resistant cell lines,[2] and two of them (KP1019 (indazolium *trans*-[tetrachlorobis-(1*H*-indazole)ruthenate(III)] and NAMI-A (imidazolium *trans*-[tetrachloro(DMSO)(imidazole)ruthenate(III)]) are in clinical trial for cancer treatment.[38]

In the present work we investigated potential factors which might be involved in the mechanism underlying the cytotoxic effects of a representative of a new class of organometallic ruthenium compounds (Ru(II)-based monofunctional complexes containing terphenyl arene ligands; i.e., the complexes fundamentally different from KP1019 or NAMI-A). We compared these factors to those involved in the mechanism underlying the cytotoxic effects of the most frequently studied metallodrug, cisplatin.

The major cytotoxic lesion induced by clinically used cisplatin is platination of DNA. One of the early effects of this platination of DNA is a reduction in the rate of DNA synthesis and a consequent slow-down in the passage of cells through the S-phase of their cycle, and subsequently, there is a dose-dependent arrest in G_2.[39] Recently, it was shown that Ru(II)-arene complexes of the type $[(\eta^6\text{-arene})\text{Ru(II)(en)Cl}]^+$ bind to DNA not only in cell free media but, even more importantly, in human cancer cell lines.[17] Moreover, it has been postulated that these monodentate Ru(II)-arene complexes belong to a class of anticancer agents for which structure−pharmacological relationships might be correlated with their DNA-binding modes.[18] Hence, based on these results, we tested the effect of monofunctional Ru(II) terphenyl complexes on the rate of DNA synthesis. Similarly to cisplatin, the Ru(II) terphenyl complexes are capable of inhibiting DNA synthesis in living cells (Figure 6). Furthermore, the inhibitory effect of cytotoxic *p*-terphenyl complex **1** was considerably more pronounced than that of the much less potent *m*-terphenyl complex **3** (Figure 6A). In addition, we have shown in our previous work[17] that cellular uptake of **1** and extent of DNA adduct formation in cells treated with **1** were markedly lower compared with the effects of **3**. Thus, the results of the present work show that in general there is no direct correlation between cellular drug uptake of monofunctional Ru(II)-terphenyl compounds or DNA adduct formation by these compounds on one hand and DNA synthesis inhibition activity on the other (Figure 6). It implies that the type of adduct formed on DNA and subsequent conformational changes may be important to the activity of this class of Ru(II)-arene compounds in tumor cells.

Cisplatin is known to exert its cytotoxic effect by inducing apoptosis.[32,40,41] To investigate further the mechanism of cell death induced by Ru(II)-arene complexes, the levels of apoptosis and necrosis were quantified. The results (Figure 2) indicate that **1** induces cell death by apoptosis in a dose-dependent manner, but with lower efficiency than cisplatin; nevertheless apoptosis prevailed over necrosis.

Activation of cell cycle checkpoints is a general cellular response after exposure to cytotoxic agents. Previous studies have indicated that cisplatin and other platinum agents predominantly inhibit cell cycle progression at S- and/or G_2/M-phase.[42,43] Our results show differences between cisplatin and Ru(II)-arene complex **1** at the level of cell cycle regulation (Figure 3). We found differences in type and dynamics of cell cycle perturbations induced by these two compounds. While cisplatin blocks A2780 cells in the G_2-phase, **1** arrests the cells in the G_0/G_1 phase. The fact that cisplatin and **1** had different effects on cell cycle progression suggests that the two drugs do not have the same cellular mechanism of action.

Since the tumor suppressor p53 plays a key role in induction of cell cycle arrest and apoptosis in response to DNA damage in human cells,[44] we examined whether the induction of cell death by **1** or cisplatin in A2780 cells was associated with changes in the level of expression of this protein. The results of this study show that treatment of human ovarian A2780 cells with **1** results in the accumulation of p53 (Figure 4, Table 2), suggesting that apoptosis induced by **1** and cell cycle arrest involves p53.

Cell cycle arrest mediated by p53 involves transactivation of cell-cycle-related factors, such as $p21^{WAF1}$. Upregulated $p21^{WAF1}$ expression is associated with cell cycle inhibition, differentiation, and cellular senescence.[45] In addition, $p21^{WAF1}$ can block DNA synthesis by binding to proliferating cell nuclear antigens.[46] As such, activation of $p21^{WAF1}$ causes subsequent arrest in the G_0/G_1 or G_2 phase of the cell cycle by interacting with the cyclin-dependent kinase complex.[47,48] Thus, the increased expression of $p21^{WAF1}$ observed after treatment of A2780 cells with **1** (Figure 5 and Table 3) is in good accord with the arrest of the cell cycle in the G_0/G_1 phase which is followed by apoptosis, as found by flow cytometric (Figure 3) and ELISA measurements (Figure 2).

It has been widely accepted that apoptosis is an active gene-directed cellular death mechanism and many different genes contribute to its regulation. Among them, proteins of Bcl-2 family draw particular attention as they represent key factors in the common final pathway involved in the regulation of apoptosis. Members of the Bcl-2 family include apoptotic antagonists (such as Bcl-2, Bcl-x, Bcl-w, etc.) and apoptotic death agonists (such as Bax, Bad, Bak, etc.). In numerous studies, elevated expression of antiapoptotic Bcl-2 family members has been associated with resistance to chemotherapy (reviewed in ref 49) *in vitro*. Resistance to cisplatin correlated positively with low levels of Bcl-2,[50] although high Bcl-2 levels confer a trend toward sensitivity.[51] In the present work, Western blot analysis revealed that **1** enhanced the expression of antiapoptotic Bcl-2 family protein Bcl-2 only very slightly, whereas expression of a proapoptotic Bcl-2 family protein Bax was markedly more upregulated (Figure 5, Table 2). As a result of these changes, the ratio of Bcl-2/Bax decreases significantly, which can trigger the apoptotic processes accompanied by DNA fragmentation, as revealed by ELISA (Figure 5, Table 2). Taken together, these findings indicate that **1**, a potent antiproliferative agent against cancer cells, is able to induce p53-mediated apoptosis in human cancer cells.

DNA adducts of metallodrugs are believed not only to mediate their cytotoxic activity in tumor cells,[2,52] but they are also mutagenic[37,53] and potentially carcinogenic.[54] Our preliminary results show a significant increase in the frequency of HPRT mutants produced in V79 cells exposed to 5 μM cisplatin, thus confirming previous results[29,37] and demonstrating that cisplatin is mutagenic. An examination of the mutation frequency data for Ru(II)-arene complexes **1**−**3** containing terphenyl ligands (Table 4) indicates that they are less mutagenic than cisplatin. Most importantly, the most cytotoxic of these Ru(II)-arene complexes, **1**, exhibited no mutagenicity, which makes this complex a suitable candidate for further preclinical and clinical testing.

In conclusion, we identified several cellular mechanisms induced by the cytotoxic Ru(II)-arene complex in cancer cells, including inhibition of DNA synthesis, overexpression and activation of p53, expression of proapoptotic proteins $p21^{WAF1}$ and Bax, G_0/G_1 arrest, and nuclear fragmentation as a result of apoptotic and, to a much lower extent, also necrotic processes.

Thus, **1** inhibits the growth of A2780 cells through induction of apoptotic cell death and G_0/G_1 cell cycle arrest. Further investigations have shown that **1** induces apoptosis by regulating the expression of the Bcl-2 family of proteins. There were significant differences in cellular responses to the treatment with monofunctional Ru(II)-complex **1** containing *para*-terphenyl arene ligand and with conventional cisplatin, particularly in the extent of these responses. In addition, the distinct p53 activation profile of the **1** compared with cisplatin provides an explanation for the activity of the monofunctional Ru(II)-complex **1** containing *para*-terphenyl arene ligand toward cisplatin-resistant cells and indicates its potential usefulness as an alternative antitumor agent for first-line or second-line therapy in patients with acquired cisplatin resistance. Our data also suggest that cytotoxic Ru(II)-arene complexes could be candidates for further evaluation as chemotherapeutic agents for human cancers, particularly in respect of their very low mutagenicity and different mode of action compared to platinum antitumor drugs in clinical use.

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