

Cytotoxic activity of novel palladium-based compounds on leukemia cell lines

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Effective treatment methods for human leukemia are under development, but so far none of them have been found to be completely satisfactory. It was recently reported that palladium complexes have significant anticancer activity as well as lower toxicity compared with some clinically used chemotherapeutics. The anticancer activities of two novel palladium(II) complexes, [Pd(sac)(terpy)](sac)·4H₂O and [PdCl(terpy)](sac)·2H₂O, were tested against three human leukemia cell lines, Jurkat, MOLT-4, and THP-1, in comparison with cisplatin and adriamycin. The cytotoxic effect of the drugs was determined using the MTT assay. Cell death was assessed using fluorescein isothiocyanate-annexin/propidium iodide staining for flow cytometry. Furthermore, p53 phosphorylation, poly(ADP-ribose) polymerase cleavage, and Bax and Bcl-2 mRNA levels were examined to elucidate the mechanism of cell death induction. Both complexes exhibited a significant dose-dependent antigrowth effect *in vitro*. The complexes predominately induced apoptosis, but necrosis was also

observed. In-vitro results have shown that palladium(II) complexes may be regarded as potential anticancer agents for treating human leukemia. Therefore, further analysis to determine the putative mechanism of action and in-vivo studies on animal models are warranted. *Anti-Cancer Drugs* 26:180–186 © 2015 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The treatment of leukemia is in constant flux, evolving and changing rapidly over the past few years. Most treatment protocols use systemic chemotherapy with or without radiotherapy. The basic strategy is to eliminate all detectable disease by using cytotoxic agents. Commonly used cytotoxic drugs include metabolic analogs, glucocorticoids, asparaginase, anthracyclines, and vincristine [1,2]. Chemotherapeutic agents kill rapidly dividing cells, thus slowing down and stopping the spread of cancerous cells. Anticancer drugs originate from a variety of sources like plants, microbes, or warfare agents. Over the past 30 years, platinum-based drugs, notably cisplatin and carboplatin, have dominated the treatment of various cancers [3]. In recent years, numerous palladium(II) [Pd(II)] complexes have been synthesized and published [4,5]. It has been shown that Pd complexes have significant anti-tumor activity to cancer cells as well as lower side effects compared with cisplatin [6]. The aqueous solubility of Pd complexes is better when compared with platinum complexes, making Pd more attractive [7]. Moreover, it is expected that Pd complexes have less kidney toxicity compared with cisplatin [8].

Ari and colleagues [9–11] synthesized and characterized two new Pd complexes, [Pd(sac)(terpy)](sac)·4H₂O (complex 1) and [PdCl(terpy)](sac)·2H₂O (complex 2) (see Figure, Supplemental digital content 1, <http://links.lww.com/ACD/A83> showing chemical structures of Pd complexes 1 and 2). The anticancer effect of these complexes 1 and 2 tested *in vitro* presents significant potential as an anticancer agent in the following cancer cell lines: A549 and H1299 for lung cancer; C6 for glioblastoma; Hep3B for liver cancer; PNT1A, PNT2-C2, BPH-1, P4E6, PC-3, and LNCaP for prostate cancer; and MCF-7 and MDA-MB-231 for breast cancer [9,10,12–14].

Complex 2 was further screened against primary cultures from seven Gleason 6/7 prostate cancer, three Gleason 8/9 prostate cancer, and four benign prostate hyperplasia patient samples, as well as against cancer stem cells selected from primary cultures. It presented considerable antigrowth effect on most primary cultures and inhibited the growth of cancer stem cells [14]. Complex 2 was found to significantly reduce the growth of tumors inoculated into mice, and presented a mild-to-low toxicity profile *in vivo* [12].

The interaction of Pd complexes with fish sperm DNA clearly indicated that these complexes act as dual-function metallointercalators and strongly bind to DNA by both intercalation and coordination [15]. Following

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exposure to complex 2, most cancer cell lines undergo apoptotic cell death, whereas cell lines A549, H1299, and PC-3 underwent necrosis [13]. Primary prostate cells induced autophagy following treatment [14]. From published data it can be concluded that Pd complexes have a differential effect on cell death that is dependent on the genetic background of the cells.

Therefore, in the present paper, we investigated the growth-inhibiting effects of complexes 1 and 2 against three different leukemia cell lines – Jurkat and MOLT-4 – as models of acute lymphoblastic leukemia and THP-1 as a model of acute monocytic leukemia.

Methods

Culture of cell lines

Human leukemia cell lines Jurkat (human acute T-lymphocyte leukemia), MOLT-4 (human acute T-lymphoblastic leukemia), and THP-1 (human acute monocytic leukemia) were used in this study. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine, and 10% fetal bovine serum (Gibco, Paisley, UK) at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced every 48 h, and cell density was maintained at 10⁶ cells/ml.

Chemicals

The drugs used in this study were cisplatin (Sigma-Aldrich), adriamycin (Farmitalia, Milan, Italy), and two novel Pd(II) complexes – [Pd(sac)(terpy)](sac)·4H₂O (complex 1) and [PdCl(terpy)](sac)·2H₂O (complex 2). The Pd complexes were synthesized at the Chemistry Department of the Science and Art, Faculty of Uludag University, Turkey, and were kindly provided by Professor Dr Veysel T. Yilmaz. The synthesis, characterization, and crystal structures have been reported previously [11]. Stock concentrations (0.2 mmol/l) of each chemical were prepared in sterile PBS and sterilized by filtration (Millipore, Billerica, Massachusetts, USA). All tests were performed by treating cells with a concentration corresponding to the IC₅₀ values for each cell line and each chemical.

MTT assay

The cytotoxic effect of chemicals was determined using MTT assay according to the modified protocol of Ulukaya *et al.* [16]. Before the assay, a stock solution of MTT (Sigma-Aldrich) was prepared (5 mg/ml PBS, pH 7.2). Cells were seeded at a density of 5000 cells/well in a 96-well plate in triplicate and grown in MEM medium (Sigma-Aldrich) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine, and 10% fetal bovine serum (Gibco), without phenol-red dye.

Cells were treated for 72 h with 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, and 51.2 µmol/l of each chemical. After the incubation period, 25 µl of MTT stock solution

was added to each well, and then after another 4 h incubation at 37°C 100 µl of solubilizing buffer (10% SDS dissolved in 0.01 N HCl) was added to each well. Cells were incubated overnight at 37°C. The absorbance at 570 nm was measured using a microplate reader (Labsystems Multiscan MS; Thermo Electron Corp., Vantaa, Finland). Untreated control cell lines were defined as 100% viable. Viability of treated cells was calculated as follows:

$$\% = 100 \frac{A(\text{treated})}{A(\text{untreated})}$$

Detection of annexin V-positive cells using flow cytometry

Cell death, following treatment, was determined using fluorescein isothiocyanate (FITC)-annexin V Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, California, USA). The kit is designed to discriminate living cells from those in the stage of early apoptosis or late apoptosis/secondary necrosis.

Following drug treatment, cells were washed twice in ice-cold PBS and centrifuged for 5 min at 800g at 4°C. They were resuspended in annexin binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 2.5 mmol/l CaCl₂, pH 7.4) to a final concentration of 2 × 10⁶ cells/100 µl. Aliquots containing 2 × 10⁶ cells in 100 µl of buffer were stained with 5 µl of FITC-annexin V [solution in 25 mmol/l HEPES, 140 mmol/l NaCl, 1 mmol/l EDTA, pH 7.4, 0.1% BSA] and 1 µl of propidium iodide (100 µg/ml in annexin binding buffer) for 15 min in the dark at room temperature. After staining, 400 µl of annexin binding buffer was added to the cells and samples were analyzed by flow cytometry within 1 h (FACScalibur; Becton Dickinson). Data analyses were performed using FACS Diva analysis software (BD BioSciences, Franklin Lakes, New Jersey, USA). Excitation was at 488 nm with an emission wavelength of 530 nm for green (fluorescein) fluorescence and 580 nm for red (propidium iodide) fluorescence.

Total protein extraction and quantification

Following treatment, cells were collected, washed twice in ice-cold PBS, and centrifuged for 5 min at 1000g, 4°C. Cells were resuspended in 150 µl of CellLytic M reagent (Sigma-Aldrich) containing a Protease Cocktail Inhibitor (Sigma-Aldrich) in the ratio 1 : 100. Samples were incubated for 30 min at 4°C on a shaker and then centrifuged for 15 min at 15 000g at 4°C. The supernatant was collected and stored at –80°C for further analysis. Protein concentrations in samples were measured using a Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer's manual. Absorbance was measured using a microplate reader (Labsystems Multiscan MS; Thermo Electron Corp.) at 570 nm.

Protein separation and immunodetection

Following protein isolation and determination of protein concentration in samples, proteins were separated by means of gel electrophoresis on SDS-PAGE 4–12% gels using a horizontal Amersham ECL Gel Box System (GE Healthcare, Life Sciences, Little Chalfont, UK). A total of 5 µg of proteins from each sample was mixed with the same volume of 2× SDS sample buffer [0.5 mol/l Tris-HCl pH 6.8, 10% (w/v) SDS, glycerol, bromophenol blue, DTT deH₂O]. Prepared samples were denatured for 5 min at 95°C. SDS-PAGE gel was prepared for electrophoresis by adding 1× running buffer (25 mmol/l Tris-HCl, 0.192 mol/l glycine, and 1% SDS in deH₂O) and prerunning it for 12 min at 160 V. Samples were then loaded into wells and electrophoresis was run for 80 min at 130 V at 4°C.

Proteins were electrotransferred to a nitrocellulose membrane (Whatman Protran, Little Chalfont, UK) using an XCell Blot module (Invitrogen) following the manufacturer's manual. Blotting pads, filter papers, and the nitrocellulose membrane were soaked in 1× transfer buffer [25 mmol/l Tris-HCl pH 7.6, 192 mmol/l glycine, 10% (v/v) methanol in mqH₂O] and gel sandwich in the cassette that was set in the tank in vertical position. The cassette was filled with 1× transfer buffer, and the tank was filled with mqH₂O. Transfer was run overnight at a constant voltage of 10 V on ice.

The membrane was blocked in 5% skimmed milk diluted in TBST buffer (1 mol/l Tris, 4 mol/l HCl, 0.06% Tween 20, deH₂O) for 1 h on a shaker at room temperature and was washed in TBST buffer and incubated overnight at 4°C with anti-poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Dallas, Texas, USA) or anti-GAPDH (Sigma Aldrich) antibodies diluted in 0.5% skimmed milk in TBST with the addition of sodium azide in the ratio 1:1000 and 1:5000, respectively. For phospho-p53 (Ser15) (Cell Signaling, Danvers, Massachusetts, USA) detection, the membrane was blocked in 5% BSA, whereas the antibody was diluted in 0.5% BSA in TBST buffer in the ratio 1:1000. The membrane was washed again in TBST buffer and incubated for 1 h at room temperature with anti-rabbit peroxidase-linked secondary antibody (ECL; GE Healthcare, Life Sciences) diluted to 1:15000 in 0.5% skimmed milk in TBST buffer. After the incubation period, the membrane was washed and dried. It was then over-layered with Amersham ECL Select Western Blotting Detection Reagent (solution A, luminol, and solution B, peroxide solution, mixed in the ratio 1:1; GE Healthcare, Life Sciences) and left in the dark for 3 min. The membrane was covered with a Hyperfilm ECL film (GE Healthcare, Life Sciences) and the film was developed using Kodak processing chemicals for autoradiography films (Sigma-Aldrich).

RNA isolation and quantitative real-time PCR

Jurkat, MOLT-4, and THP-1 untreated cells were harvested after 24 and 72 h in culture, whereas treated samples were harvested after 72 h incubation period. Total RNA was isolated using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Total RNA mass of 4 µg was treated with DNase I (Thermo Scientific) added in 10× reaction buffer with MgCl₂ (Thermo Scientific) and EDTA. DNase I-treated RNA was then used to synthesize cDNA using 10× RT-PCR buffer, MgCl₂, and oligo dNTPs from the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, California, USA), as well as Ribolock RNase Inhibitor (Thermo Scientific), random hexamers (Invitrogen), and MuLV Reverse Transcriptase (Roche Applied Science Painsberg, Germany). Reverse transcription was performed in a thermomixer (Eppendorf, Hamburg, Germany) at the following conditions: 10 min at 20°C, 1 h at 42°C, 5 min at 99°C, and 5 min at 5°C. Gene expression levels were determined by means of quantitative reverse transcription-PCR using Power SYBR Green Mastermix (Applied Biosystems). The following primers were used to determine gene expression levels: human *Bcl-2* forward primer 5'-GATTGTGGCCTTCTTTGAG, melting temperature 59.8°C; human *Bcl-2* reverse primer 5'-GTTCCACAAAGGCATCC, melting temperature 59.0°C; human *Bax* forward primer 5'-AACTGGACA GTAACATGGAG, melting temperature 56.6°C; human *Bax* reverse primer 5'-TTGCGGCAAAGTAGAAAAG, melting temperature 59.7°C; human *ACTB* forward primer 5'-GACGACATGGAGAAAATCTG, melting temperature 59.7°C; and human *ACTB* reverse primer 5'-ATGATCTGGGTCATCTTCTC, melting temperature 58.0°C (Sigma-Aldrich). The PCR reaction conditions were as follows: 10 min at 95°C for one cycle, 15 s at 95°C, and 1 min at 60°C for 40 cycles. Quantitative reverse transcription-PCR was performed on a 7500 Fast PCR system (Applied Biosystems).

Statistical analysis

All results are presented as mean ± SD. Significant differences between groups were evaluated by means of Student's *t*-test ($P < 0.05$).

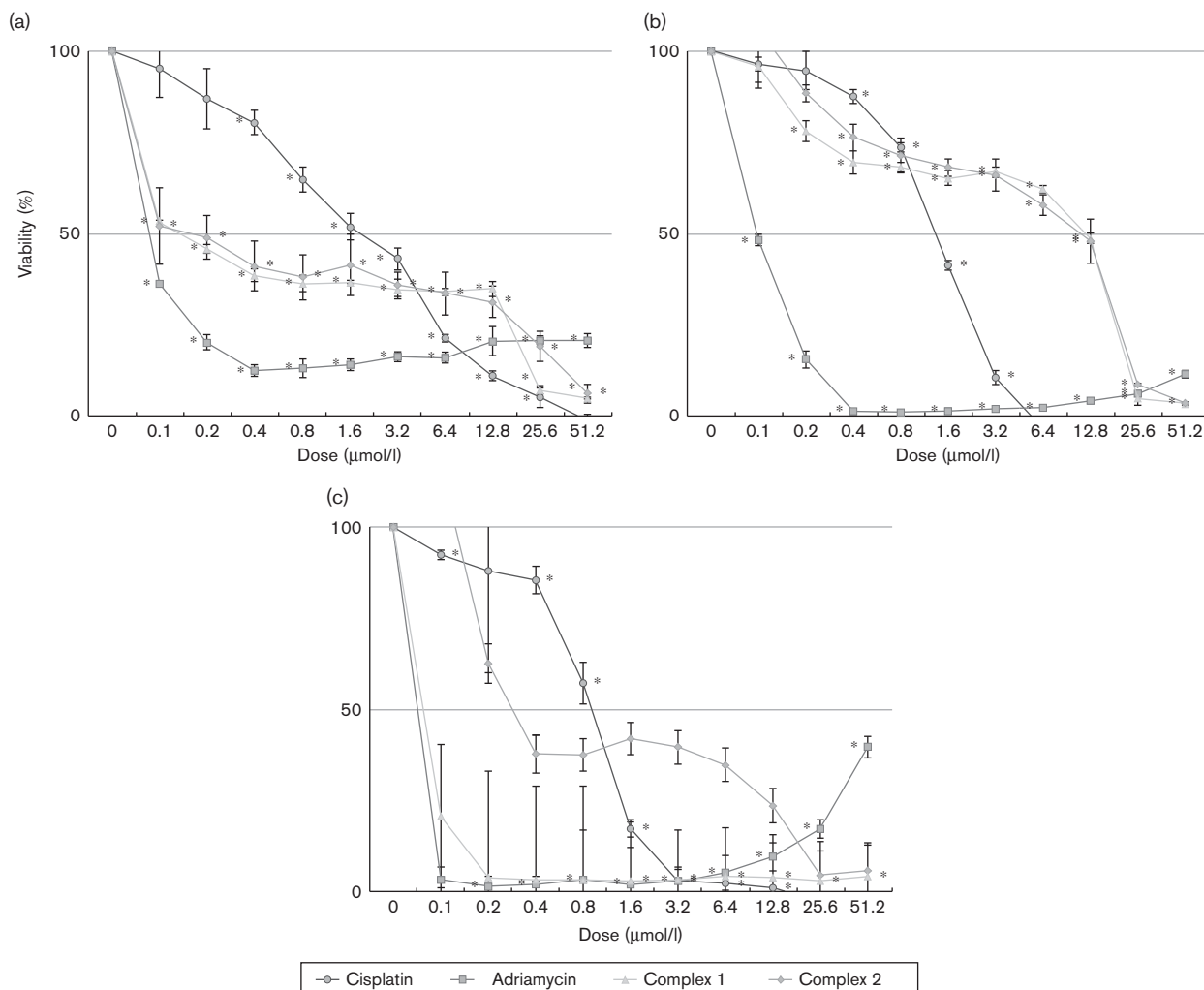
Results

Growth-inhibiting effects of the complexes, cisplatin, and adriamycin

The antigrowth effects of complexes 1 and 2, cisplatin, and adriamycin on leukemia cell lines (Jurkat, MOLT-4, and THP-1) were evaluated using the MTT assay after treating cells with different concentrations of the tested compounds for 72 h.

Both Pd complexes significantly inhibited the growth of leukemia cells in a dose-dependent manner (Fig. 1). However, the extent of cell sensitivity to the tested compounds varied among the cell lines used. Adriamycin

Fig. 1



Growth-inhibiting effects of palladium complexes 1 and 2, cisplatin, and adriamycin in Jurkat (a), MOLT-4 (b), and THP-1 (c) cell lines were determined using MTT viability assay 72 h after treatment. The doses used ranged from 0.1 to 51.2 µmol/l. Significant differences in comparison with untreated control ($P < 0.05$) are marked with asterisks.

Table 1 The IC₅₀ values of the cisplatin, adriamycin, and palladium complexes 1 and 2

Cell line	Treatment	IC ₅₀ (µmol/l)
Jurkat	Cisplatin	1.94
	Adriamycin	0.08
	Complexes 1	0.14
	Complexes 2	0.18
MOLT-4	Cisplatin	1.38
	Adriamycin	0.1
	Complexes 1	12.22
	Complexes 2	11.45
THP-1	Cisplatin	0.94
	Adriamycin	0.05
	Complexes 1	0.06
	Complexes 2	0.24

was found to be superior in all three leukemia cell lines, whereas cisplatin was the least effective in Jurkat and THP-1 cell lines. Jurkat and MOLT-4 cell lines were

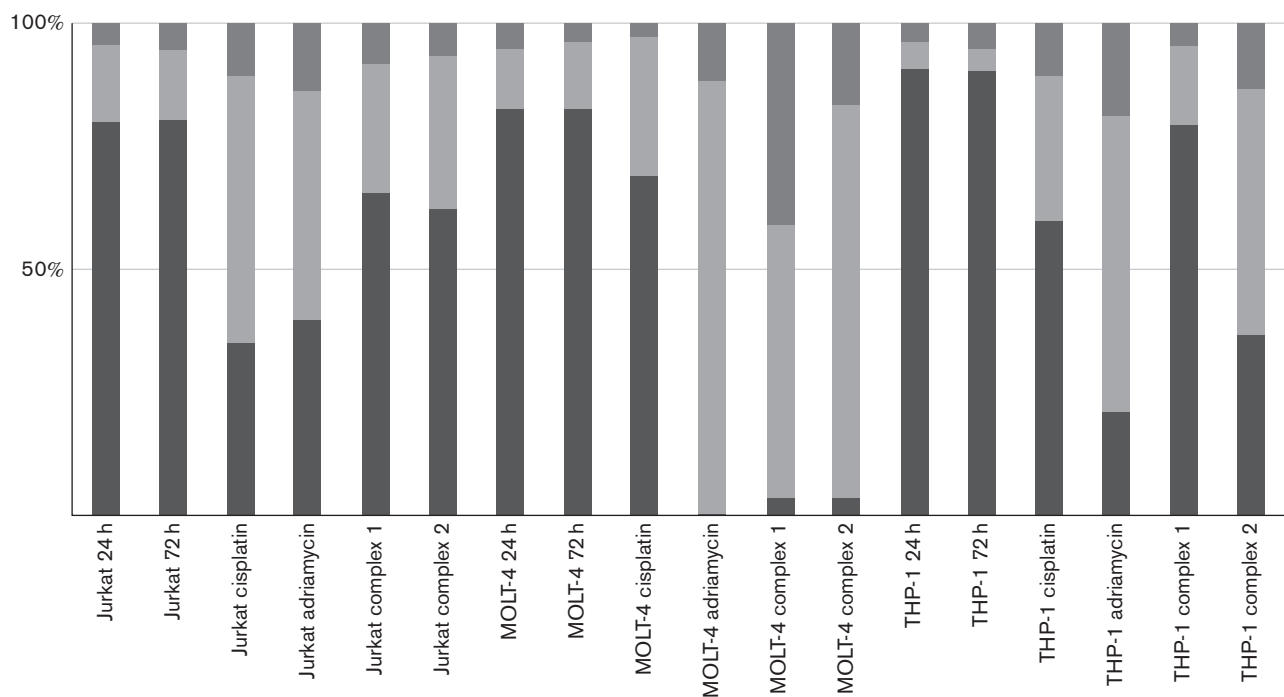
equally sensitive to complexes 1 and 2, whereas the THP-1 cell line was more sensitive to complex 1 than to complex 2.

Following the MTT assay, IC₅₀ values were calculated (Table 1). For the Jurkat and THP-1 cell lines, IC₅₀ values of complexes 1 and 2 were between IC₅₀ values of cisplatin and adriamycin, presenting better antigrowth effect compared with platinum-based cisplatin. IC₅₀ values for complexes 1 and 2 for the MOLT-4 cell line were the highest.

Determination of cell death using annexin V staining

The cell death-inducing effects of complexes 1 and 2, cisplatin, and adriamycin were evaluated by flow cytometry to detect phosphatidylserine on the outer membrane of cells marking apoptosis. Jurkat, MOLT-4, and

Fig. 2



Flow cytometric analysis of cell death in Jurkat, MOLT-4, and THP-1 cell lines after treatment with complexes 1 and 2, cisplatin, and adriamycin for 72 h. Apoptosis is determined by prominent annexin V and low PI staining. Summary of analysis is represented in columns showing proportion of viable (dark gray), early apoptotic (light gray), and late apoptotic/necrotic (gray) cells. PI, propidium iodide.

THP-1 cell lines were treated with a compound concentration of IC_{50} value. The treatments resulted in cell death induction in all cell lines (Fig. 2). The Jurkat cell line presented the best apoptotic response to cisplatin and adriamycin. The viability was decreased after treatment with Pd complexes, and apoptosis was the dominant type of cell death. The MOLT-4 cell line presented the best apoptotic response to adriamycin. The viability was very low after treatment with Pd complexes. Apoptosis was the dominant type of cell death, but the level of secondary necrosis was also significant. The THP-1 cell line presented the best response to adriamycin and complex 2, and the cell death included apoptosis and secondary necrosis (see Figure, Supplemental digital content 2, <http://links.lww.com/ACD/A84> showing flow cytometry analysis).

Apoptosis induction through the intrinsic pathway

Apoptosis is activated through the phosphorylation of p53, which signals DNA damage. Phosphorylation of p53 is present and induced in all three leukemia cell lines after treatment with complexes 1 and 2, cisplatin, and adriamycin. The MOLT-4 cell line was treated with the highest concentrations of drugs corresponding to IC_{50} values, causing quick cell death and protein degradation, which was judged by the disappearance of the full-length PARP-1 bands.

The executor of apoptosis is caspase 3, the protease that cleaves PARP-1 among other substrates. Therefore, cleavage of PARP-1 is a marker of apoptosis. PARP-1 was cleaved following treatment in all three leukemia cell lines; however, the extent was different (Fig. 3). The Jurkat cell line showed slight amount of cleavage after treatment with complexes 1 and 2. The MOLT-4 cell line showed full-length PARP-1 protein degradation possibly due to high concentrations of complexes 1 and 2. The THP-1 cell line showed slight PARP-1 cleavage after treatment with cisplatin and complex 1 and extensive PARP-1 degradation after treatment with adriamycin and complex 2.

Changes in mRNA levels of two genes of the *Bcl-2* family were simultaneously analyzed, *Bax* as a proapoptotic and *Bcl-2* as an antiapoptotic member, to elucidate the cell death pathway (Fig. 4). The Jurkat cell line responded to classical treatments, to cisplatin, and to adriamycin, with increased levels of both *Bax* and *Bcl-2*, whereas treatment with complex 1 resulted in an increase in *Bax* and decrease in *Bcl-2* (Fig. 4a).

Treatment of the THP-1 cell line with any of the drugs/complexes did not result in significant changes in gene expressions because the change did not occur two-fold or higher (Fig. 4b).

Fig. 3

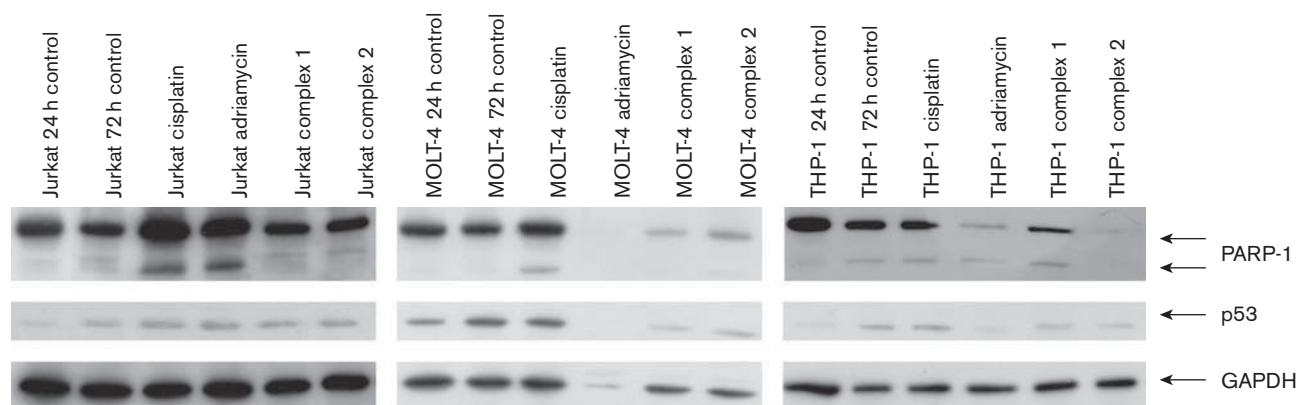
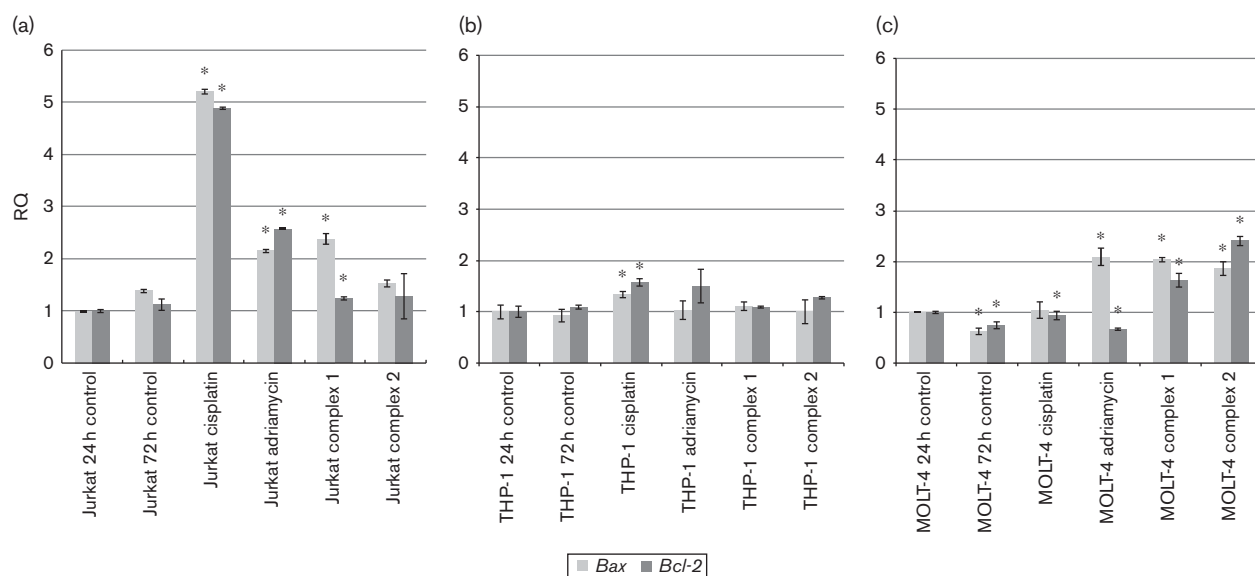


Fig. 4



Treatment of the MOLT-4 cell line with adriamycin and complex 1 resulted in an ideal response, which is characterized by an increase in *Bax* and decrease in *Bcl-2*. However, neither cisplatin nor complex 2 showed such an effect (Fig. 4c).

Discussion

In this study, the cytotoxic activity of two novel Pd(II) complexes, which seem to be promising anticancer complexes against solid tumors, has been determined.

These complexes were previously studied on some solid tumors as explained in the previous sections of this paper and satisfactory results were reported [10,12–14]. Therefore, we suspected their similarly potent activities against leukemic malignancies. In fact, we obtained promising results for these malignancies. Adriamycin, which is one of the classical drugs used for the treatment of leukemic malignancies, was used as a reference drug to compare the results with these complexes [17]. Cisplatin, a platinum-based compound, with DNA intercalating

mechanism of anticancer action, similar to a platinum-based compound, was used as another reference drug [18]. In particular, complex 1 yielded comparable results with adriamycin in two cell lines, Jurkat and THP-1. The IC₅₀ values were similar to each other in these tumor types (Table 1). It was 0.08 and 0.05 for adriamycin and 0.14 and 0.06 for complex 1 in Jurkat and PTH-1 cells, respectively. These results warrant complex 1 for further in-vivo analysis.

Further, the mode of cell death was studied using flow cytometry (Fig. 2). In these two cell lines, early apoptosis was induced by both adriamycin and complex 1, but the amplitude of the increase was better in adriamycin than in complex 1, which shows that complex 1 might induce other types of cell death as well. In fact, in Jurkat cells the cleavage of PARP-1 was more pronounced compared with THP-1 cells. In THP-1 cells (as well as in MOLT-4 cells), full-length PARP-1 actually almost disappeared possibly because of high protein degradation by the complexes, especially by complex 2 in MOLT-4 cells. This may be explained by the effect of complex 2 on autophagy that is a process of macromolecule degradation in cells. Ulukaya *et al.* [14] showed that complex 2 also induced autophagy in prostate cancer cells.

In terms of the mechanism of action, mitochondria seem to play a role in the cytotoxic activity of these complexes. For example, it was found that complex 1 increased the ratio of *Bax/Bcl-2* in Jurkat cells as well as in MOLT-4 cells (Fig. 4). The increase in this ratio is a well-known action in apoptosis [19]. In fact, PARP-1 cleavages confirm apoptosis in Jurkat cells as well as the presence of the DNA damage-activated Ser15-phosphorylated form of p53 [20,21]. The similar PARP-1 and p53 pattern was present in MOLT-1 and THP-1 cell lines (Fig. 3).

Taken together, it seems that complexes 1 and 2 have comparable and promising activities with the classical antileukemic drug adriamycin and the similar platinum-based drug cisplatin. Therefore, in-vivo experiments are warranted.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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