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Biological evaluation of novel Pt(II) and Pd(II) complexes with pyrazole-containing ligands

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Abstract

The new platinum (II) and palladium (II) complexes (**2**–**4**) with ligands 5-(2-hydroxyphenyl)-1,3-dimethyl-4-(dimethoxy)phosphonyl-1*H*]-pyrazole (**1a**) and 5-(2-hydroxyphenyl)-1,3-dimethyl-4-methoxycarbonyl-1*H*]-2-pyrazole (**1b**) were screened in a search for novel anticancer agents. Thus, alkylating activity, cytotoxicity, ability for induction of apoptosis and binding to DNA were tested. The *cis*-[Pt(**1b**)$_2$Cl$_2$] complex (**3b**) was the most potent alkylating agent in a Preussmann test, in comparison with the other test compounds and *cis*-platin. The highest cytotoxicity against the HL-60 and NALM-6 leukemia cell lines was observed for complexes **3b** and **4b** (*trans*-[Pd(**1b**)$_2$Cl$_2$]), although the extent of the effect was lower relative to *cis*-platin. Moreover, both complexes were remarkably less toxic to human umbilical vein endothelial cells (HUVECs) with IC_{50} values of **3b** 14 and 20 times higher than that ones for HL-60 and NALM-6 cells, respectively. Complexes **3b** and **4b** induced caspase-3 activity. Apoptosis occurred in a strictly dose-dependent manner and required only low concentrations of **4b**. However, compounds **3b** and **4b** showed lower binding affinity to double-stranded DNA than *cis*-platin.

Keywords: Pyrazole ligand; Palladium (II) complex; Platinum (II) complex; Alkylating activity; Cytotoxic effect; Apoptosis

1. Introduction

Cis-platin [diamminedichloroplatinum, *cis*-$PtCl_2(NH_3)_2$] is known as a DNA-modifying agent with strong anticancer potency (Reedijk, 1999; Jakupec et al., 2003; Jamieson and Lippard, 1999). Despite its wide application as a therapeutic agent in chemotherapy, *cis*-platin is associated with many serious side effects, such as nephrotoxicity, ototoxicity and allergy (Garnuszek et al., 2002). Thus, various platinum (II) and palladium (II) complexes with nitrogen-containing ligands are the subject of intensive biological evaluations in the search for less toxic and more selective anticancer therapeutics (Wong and Giandomenico, 1999, Jakupec et al., 2003). Among them, the class of pyrazole-containing complexes has been reported to possess antitumor activity comparable to that of *cis*-platin (Sakai et al., 2000, Wheate et al., 2001; Al-Allaf and Rashan, 2001). In addition, considerable interest in the pyrazole moiety has been stimulated by promising pharmacological, agrochemical and analytical applications of pyrazole-containing derivatives (Elguero, 1996, Eicher and Hauptmann, 1995, Onoa et al., 1999, Onoa and Moreno, 2002). Recently, substituted pyrazoles have been used as analytical reagents in the complexation of transition metal ions (Wisniewski et al., 1994, Chruscinski et al., 2000).

For many years, our research has been focused on the synthesis of phosphonic derivatives of chromone and the evaluation of their biological properties in the search of

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active anticancer agents (Budzisz et al., 2002). We recently described the synthesis of 5-(2-hydroxyphenyl)-1,3-dimethyl-4-(dimethoxy)phosphonyl-1*H*]-pyrazole (**1a**) and 5(2-hydroxyphenyl)-1,3-dimethyl-4-methoxycarbonyl-1*H*]-2-pyrazole (**1b**) which we used as ligands (L) in the formation of ML_2Cl_2 complexes with platinum (II) and palladium (II) metal ions (M) (Fig. 1) (Budzisz et al., 2004).

We assumed that dimethoxyphosphonyl and/or methoxycarbonyl moieties present in ligand residues **1a** and **1b**, respectively, would be good alkylating agents, while highly substituted pyrazole ligands may confer Pt(II) and Pd(II) complexes **2**–**4** higher cytotoxicity and better selectivity toward cancer cells as compared to *cis*-platin.

Here we present data on the biological evaluation of these new platinum (II) and palladium (II) complexes **2**–**4**, including their alkylating activity, cytotoxicity and ability for induction of apoptosis and binding to DNA.

2. Materials and methods

2.1. Chemicals

All reagents and solvents used were of analytical grade.

2.2. Preparation of the complexes

Potassium tetrachloroplatinate (II) and bis(benzonitrile)-dichloropalladium (II) were used as metal ion reagents. The synthesis of ligands and complexes was carried out as described (Budzisz et al., 2004).

2.3. Determination of alkylating properties (Preussmann test)

The test compound (0.005 mmol) was dissolved in 2-methoxyethanol (1 ml) and a solution of 4-(4′-nitrobenzyl)-

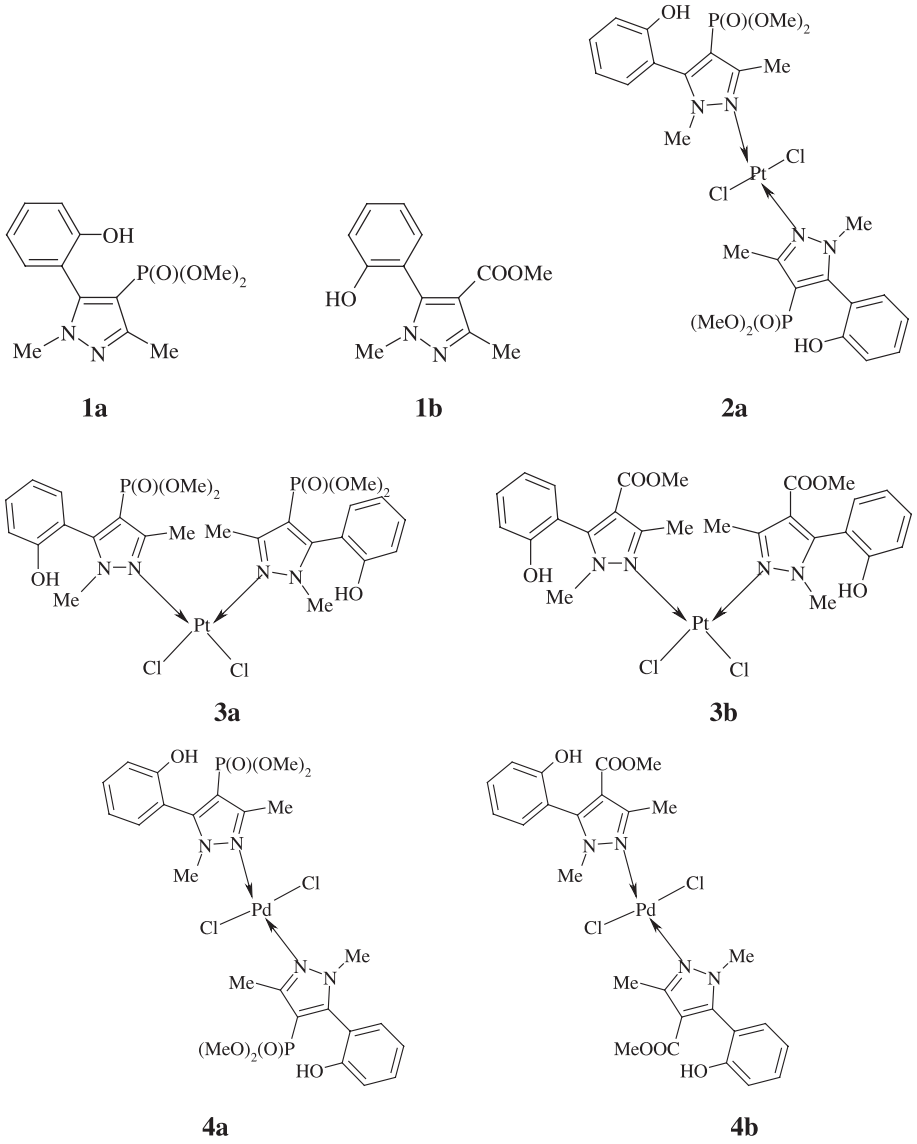

Fig. 1. The chemical structures of the pyrazole ligands and their platinum (II) and palladium (II) complexes.

pyridine (NBP) in 2-methoxyethanol (5% solution, 1ml) was added. The sample was heated at 100±0.5 °C for 1 h and then quickly cooled to 20 °C. 2-Methoxyethanol (2.5 ml) and piperidine (0.5 ml) were added to the sample to give a total volume of 5 ml. The final concentration of the test compound was 5×10^{-4} to 1×10^{-3} M. After 90 s, the absorbance was measured at $\lambda_{560\ nm}$ in a glass cell (1 cm). 2-Methoxyethanol ethanol was used as a reference solvent.

2.4. Cells and cytotoxicity assay

Human umbilical vein endothelial cells (HUVECs) as well as human leukemia promyelocytic HL-60 and lymphoblastic NALM-6 cell lines were used. HUVEC cells were isolated from freshly collected umbilical cords as previously described (Jaffe et al., 1973), and cultured in plastic dishes coated with gelatin, in RPMI 1640 medium supplemented with 20% FBS (foetal bovine serum), 90 U/ml heparin, 150μg/ml endothelial cell growth factor (ECGF, Roche Diagnostics, Mannheim, Germany) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin). Cells were grown in monolayer at 37 °C in an atmosphere of 5% CO_2 (Jaffe et al., 1973).

The NALM-6 cell line was purchased from the German Collection of Microorganisms and Cell Cultures. Cells were cultured in RPMI-1640 medium (developed by Moore and Woods, 1976, Roswell Park Memorial Institute) supplemented with 10% foetal calf serum in a 5% CO_2–95% air atmosphere. Exponentially growing cells were seeded at 3×10^5/well on 24-well plate (Nunc), and cells were then exposed to the test compounds for 48 h. Stock solutions of test compounds were freshly prepared in dimethylsulfoxide (DMSO), then dilutions from 10^{-3} to 10^{-7} M in complete culture medium were made.

For HL-60 and NALM-6 cells, the number of viable cells was counted in a Buerker hemocytometer using the trypan-blue exclusion assay (Budzisz et al., 2003). The values of IC_{50} (the concentration of test compound required to reduce the cell survival fraction to 50% of the control) were calculated from dose–response curves and used as a measure of cellular sensitivity to a given treatment. All data are expressed as means ± S.D.

For HUVEC cells, the cytotoxicity of **3b**, **4b** and *cis*-platin was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO] assay as described (Maszewska et al., 2003). Briefly, after 46 h of incubation with drugs, the cells were treated with the MTT reagent and incubation was continued for 2 h. MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH 4.7 and absorbance was read at 562 and 630 nm on an ELISA-PLATE READER (ELX800, Bio-Tek, USA). Complexes **3b**, **4b** and *cis*-platin were tested for their cytotooxicity in a final concentration 10^{-3}–10^{-7} M. As a control, cultured cells were grown in the absence of complexes. Data points represent means of at least 12 repeats.

2.5. Caspase-3 activity assay

HL-60 and NALM-6 cells (2×10^6) were treated with compounds **3b**, **4b** and formazan and HL-60 cells (2×10^6) were treated *cis*-platin at concentrations $5.0\times IC_{50}$, $1.0\times IC_{50}$ and $0.2\times IC_{50}$. After 1, 2 and 5 h of incubation, the cells were spun, washed twice with cold 0.01 M phosphate buffer containing 0.9% NaCl and lysed with dithiothreitol. Cellular lysates were used directly for determination of enzyme activity. Caspase-3 activity assay, based on the capture of caspase-3 from cellular lysates by a monoclonal antibody, was done according to the manufacturer's protocol (Roche). Caspase-3 activity is proportional to the developed fluorochrome (amidofluorocoumarin, AFC) released from the substrate [acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC)]. Generated free AFC was determined fluorometrically at $\lambda_{505\ nm}$ (Victor-2). Enzyme activity is expressed as the concentration (in μM) of AFC released by 10^6 cells.

2.6. Isolation of plasmid DNA

The pBluescript II SK+ plasmid (Stratagene) was isolated from *Escherichia coli* strain DH5α according to the alkaline lysis method (HiSpeed Plasmid Midi Kit, Qiagen). Electrophoretic scanning of the DNA sample in an agarose gel showed that 90% of the plasmid was in the covalent closed circular form and 10% in the open circular form.

2.7. Digestion of drug-modified plasmid DNA with restriction endonucleases

DNA (0.6 μg) was incubated with the test compound (drug) in the platination buffer (3 mM NaCl, 1 mM Na_2HPO_4, pH 7.4) for 18 h at 37 °C (Bancroft et al., 1990). To separate drug-modified plasmid DNA from unbound drug, phenol extraction and ethanol precipitation were performed prior to the enzymatic digestion.

The drug-modified DNA was digested with either *Hin*dIII (Promega) or *Bam*HI (Promega) restriction endonucleases in appropriate buffers until completion. Products of the reaction were subjected to 1% agarose gel electrophoresis for 2 h at 80 V in TBE buffer. After ethidium bromide staining, the gels were photographed under a UV lamp (BioRad, GelDoc 2000). Densitometry of the cut DNA fraction versus uncut DNA fractions was performed to measure the affinity of the drug toward the restriction site DNA sequences. All experiments were repeated at least four times.

3. Results

3.1. Alkylating activity of complexes *2–4* and their ligands *1a* and *1b*

The alkylating activity of ligands **1a** and **1b** (Fig. 1A) as well as their platinum (II) (**2a**, **3a**, **3b**) and palladium (II)

Table 1
Alkylating activity of highly substituted pyrazoles and their Pt(II) and Pd(II) complexes

Compound	Molar extinction coefficient (ε)	Absorbance (A)[a], λ_{max}=560 nm	Alkylation activity[b]
1a	192.8	0.1928	++
1b	49.6	0.0496	–
2a	170.7	0.1707	++
3a	282.2	0.2822	++
3b	544.1	0.5441	+++
4a	181.2	0.1812	++
4b	65.6	0.0656	+
cis-Platin[c]	300.0	0.300	++

[a] Means from three determinations.
[b] According to Preussmann (Preussmann et al., 1969); (–) A<0.05, (+) A=0.05–0.1, (++) A=0.1–0.5, (+++) A>0.5.
[c] According to Zyner et al. (1999).

complexes (**4a**, **4b**) was determined according to the Preussmann test (Preussmann et al., 1969). The screening of ligands **1** and complexes **2**–**4** was carried out in 2-methoxyethanol at a concentration of 0.005 mmol/ml. The resultant data are collected in Table 1. The *cis*-[Pt(**1b**)$_2$Cl$_2$] complex (**3b**) is the most potent alkylating agent, relative to *cis*-platin and the other test compounds. It is twice as potent an alkylating agent as the *cis*-[Pt(**1a**)$_2$Cl$_2$] complex (**3a**). The *trans*-complexes **2a** and **4a**, *trans*-[Pt(**1a**)$_2$Cl$_2$] and *trans*-[Pd(**1a**)$_2$Cl$_2$], respectively, exhibit only moderate alkylating activity.

3.2. Cytotoxicity of complexes 2–4 and ligands 1a and 1b

Complexes **2a**, **3a, 3b**, **4a** and **4b** and their ligands **1a** and **1b** were at first evaluated for cytotoxicity against the two human leukemia cell lines, HL-60 and NALM-6. *Cis*-platin and carboplatin were used as references. The IC$_{50}$ values were determined for a broad range of drug concentration, from 10^{-7} to 10^{-3} M (Table 2).

The *trans*-[Pd(**1b**)$_2$Cl$_2$] complex (**4b**) exhibited the highest toxicity towards HL-60 and NALM-6 cells with IC$_{50}$ coefficients 25.7 and 8.9 μM, respectively. These values are several times lower than that of the reference *cis*-platin. The *cis*-platinum complex **3b** showed remarkable cytotoxicity to both cell lines, while the Pt(II) and Pd(II) *trans*-complexes **2a** and **4a** showed very low cytotoxicity, with IC$_{50}$ values above 500 μM.

Table 2
Cytotoxic activity of ligands **1a** and **1b** and their Pt(II) and Pd(II) complexes **2**–**4**

Compound	HL-60 IC$_{50}$ (μM)	NALM-6 IC$_{50}$ (μM)	HUVEC IC$_{50}$ (μM)
1a	>1000	594.0±26.0	–
1b	451.0±34.0	495.0±75.0	–
2a	535.0±46.0	528.0±23.0	–
3a	435.0±34.0	271.0±64.0	–
3b	51.6±4.7	36.5±13.3	708.7±28.0
4a	494.0±65.0	515.0±38.0	
4b	25.7±0.9	8.9±1.3	92.4±3.8
cis-Platin	0.8±0.12	0.7±0.3	96.0±5.7
Carboplatin	4.3±1.3	0.7±0.2	–

Moreover, complexes **3b** and **4b** tested in a normal non-cancerogenic HUVEC cells were remarkably less toxic. The values of IC$_{50}$ of **3b** were 14 and 20 times higher and of **4b**–4 and 10 times higher than those for HL-60 and NALM-6 cells, respectively. Thus, the effectiveness of **3b** was significantly higher than of **4b**.

3.3. Induction of programmed cell death by compounds 3b and 4b

The most cytotoxic complexes **3b** and **4b** were tested for their ability to induce caspase-3 activity in HL-60 and NALM-6 cells. For comparison, *cis*-platin was used as a reference for a caspase-3 activation assay in HL-60 cells. In this assay, cells were treated with the test compounds in three different concentrations (0.2×IC$_{50}$, 1.0×IC$_{50}$ and 5.0×IC$_{50}$). The time course of the induction of caspase-3 activity, expressed as a concentration of released AFC reagent, is shown in Figs. 2, 3 and 4. The induction of

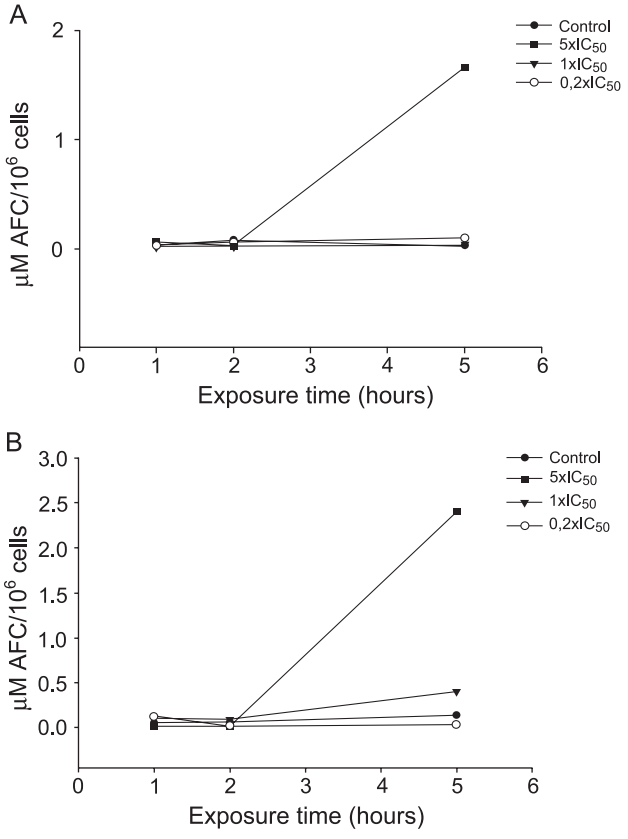

Fig. 2. Time course of induction of caspase-3 activity by *cis*-[Pt(**1b**)$_2$Cl$_2$] complex **3b** in (A) HL-60 cells and (B) NALM-6 cells. Test cells were treated with **3b** at the concentration 5.0×IC$_{50}$, 1.0×IC$_{50}$ and 0.2×IC$_{50}$ (μM). After 1, 2 and 5 h of incubation time, the cells were lysed and quantified for caspase-3 activity, which is proportional to the amidofluorocoumarin (AFC) released from the labelled substrate. Generated free AFC was determined fluorometrically at $\lambda_{505\ nm}$. Enzyme activity is expressed as the concentration (in μM) of AFC released by 10^6 cells.

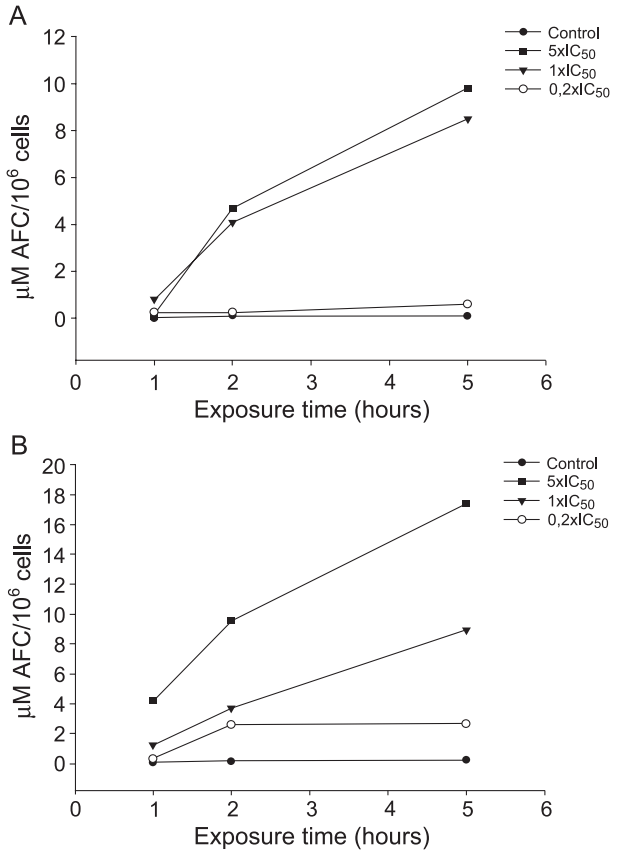

Fig. 3. Time course of induction of caspase-3 activity by *trans*-[Pd(**1b**)$_2$Cl$_2$] complex **4b** in (A) HL-60 cells and (B) NALM-6 cells. Test cells were treated with **4b** at the concentration 5.0×IC$_{50}$, 1.0×IC$_{50}$ and 0.2×IC$_{50}$ (µM). After 1, 2 and 5 h of incubation time, the cells were lysed and quantified for caspase-3 activity as described above.

caspase-3 activity begins rapidly within 2 h after exposing the cells to Pd(II) complex **4b** and continues in a strictly dose-dependent manner. This effect is seen in both test cell lines. In the case of Pt(II) complex **3b**, initiation of the apoptosis process is slower and observed only at a high drug concentration (5×IC$_{50}$). In contrast, when HL-60 cells are exposed to *cis*-platin, caspase-3 activity is induced after 1 h. However, the extent of the effect is much lower than that caused by **4b** when cells are exposed to high drug concentration (5×IC$_{50}$) for a long time (5 h).

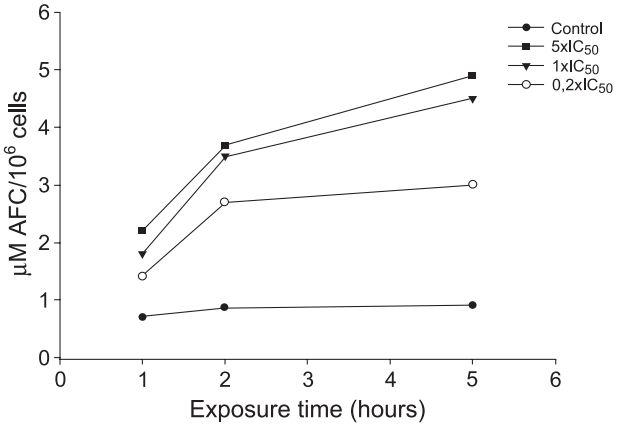

Fig. 4. Time course of caspase-3 activity induction by *cis*-platin in HL-60 leukemia cells.

3.4. Digestion of drug-modified plasmid DNA with restriction endonucleases

To study the sequence-specific affinity of **3b** and **4b** towards double-stranded DNA, analysis with restriction endonucleases was performed. *Cis*-platin, which binds to DNA preferentially at the GG sequences (Jung et al., 2001), was used as a reference. Two restriction sites, *Bam*HI (G↓GATCC) and *Hin*dIII (A↓AGCTT), were chosen to analyze the pattern of binding of **3b** and **4b** complexes to DNA. Both restriction sites are unique in the pBluescript SK+II DNA plasmid. Only covalent modifications were studied.

The pattern of DNA bands obtained after digestion of the pBluescript SK+II DNA plasmid DNA and the drug-DNA derivative with restriction enzymes *Bam*HI or *Hin*dIII is shown in Fig. 5. As expected, for the *cis*-platin-modified plasmid DNA its digestion by *Bam*HI was significantly more inhibited than by *Hin*dIII. Densitometric analysis of the gel showed that the cutting capacity of *Bam*HI was decreased to 40.4% and 31.3%, whereas *Hin*dIII activity only to 92.6% and 85.8% when *cis*-platin was used at concentrations of 10 and 50 µM, respectively (Table 3). Inhibition of the cutting capacity of *Bam*HI enzyme by either **3b** and **4b** is lower than that of *cis*-platin. The ligand molecule in the derivatives **3b** and **4b** seems to decrease the affinity of the metal complex towards the G·C regions. The ligand itself does not influence the ability of both enzymes to cut the DNA. The lower ability of **3b** and **4b** to inhibit *Bam*HI activity, in comparison to *cis*-platin, may be due to

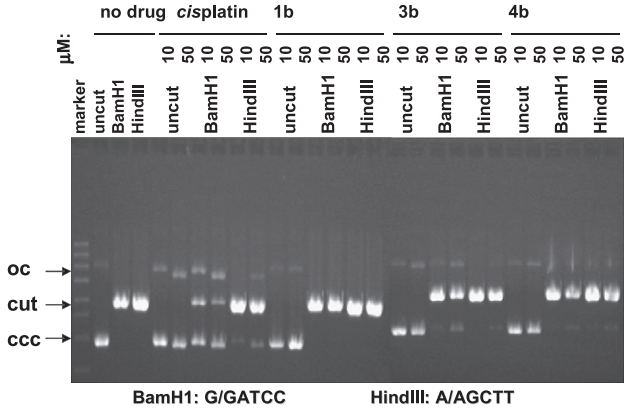

Fig. 5. *Bam*HI and *Hin*dIII digestion of drug-modified DNA. Two concentrations of drugs were used in the study: 10 and 50 µM. In the control experiment (lanes 2–4), native plasmid DNA was incubated in platination buffer in the absence of drugs. Electrophoresis in 1% agarose gel following digestion was performed. Densitometry of the cut DNA fraction versus uncut DNA fractions (ccc and oc) is summarized in the Table 3; ccc, covalent closed circular plasmid DNA; oc, open circular plasmid DNA.

Table 3
Cutting capacity of *Bam*HI and *Hin*dIII restriction endonucleases by platinum (II) and palladium (II) complexes

Enzyme	*Bam*HI		*Hin*dIII	
	Drug concentration (μM)			
	10	50	10	50
cis-Platin	40.4±6.2[a]	31.3±6.5	92.6±6.7	85.8±0.6
3b	79.5±7.9	65.7±6.4	95.8±5.0	92.0±3.5
4b	76.0±10.8	51.7±14.6	88.8±8.4	76.0±7.5
1b	100±0	100±0	100±0	100±0

[a] The numbers indicate the percent (%) of plasmid DNA in the cut fraction. The experiment was performed under conditions where the control plasmid DNA was cut completely in the absence of drugs. Each experiment was repeated at least four times and the standard deviation was calculated.

the decreased affinity of these new complexes towards double-stranded DNA.

4. Discussion

In the framework of a search for new *cis*-platin analogs which might serve as less toxic and more selective anticancer therapeutics, we have investigated the alkylating, cytotoxic and apoptic activity of the novel platinum (II) and palladium (II) complexes **2**–**4** of the highly substituted pyrazole-containing ligands **1a** and **1b** (Budzisz et al., 2004) (Fig. 1).

Although the alkylating activity of ligand **1a,** containing a methyl phosphonic group, was much higher than its carboxylic analog **1b**, the *cis*-[Pt(**1b**)$_2$Cl$_2$] complex **3b**, was the most potent alkylating agent relative to *cis*-platin and the other test compounds. For complexes containing ligand **1a**, the alkylating activity was in the same range as for the ligand. This suggests that, for the methyl carboxyl-group-containing complex **3b**, its alkylating activity is rather a feature of the complex and not the ligand. Surprisingly, *trans*-[Pd(**1b**)$_2$Cl$_2$] (**4b**), exhibiting the lowest alkylating activity, was the most cytotoxic of the test compounds towards HL-60 and NALM-6 cells, although its cytotoxicity was significantly lower than *cis*-platin and carboplatin. Complexes **3b** and **4b**, the most cytotoxic to human leukemia cell lines, both showed remarkably lower toxic effects in the non-cancerogenic HUVECs. This feature might be of interest in respect to effectiveness of the potential drugs, which should exhibit a high ratio of the IC$_{50}$ values for non-cancerogenic to cancer cells. The high cytotoxic activity of **3b** and **4b** enabled us to test whether these complexes induce irreversible cell death via the caspase-3 activation pathway. Interestingly, the *trans*-[Pd(**1b**)$_2$Cl$_2$] complex **4b** activated caspase-3 in a strictly dose-dependent manner in NALM-6 and HL-60 cell lines. Moreover, NALM-6 cells seemed to be more sensitive to such treatment than HL-60 cells. In the case of complex **3b**, initiation of the apoptosis process was much slower and needed a higher drug concentration. As shown by restriction endonuclease analysis, the sequence-specific affinity of **3b** and **4b** towards double-stranded DNA was significantly lower than that of *cis*-platin. Thus, activation of the caspase-3 pathway probably occurs via a mechanism different from DNA damage.

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