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ORIGINAL ARTICLE

In vitro evaluation of dichloro-bis(pyrazole)palladium(II) and dichloro-bis(pyrazole)platinum(II) complexes as anticancer agents

Frankline K. Keter · Stonard Kanyanda · Sylvester S. L. Lyantagaye · James Darkwa · D. Jasper G. Rees · Mervin Meyer

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Abstract
Introduction Cisplatin (*cis*-diamminedichloroplatinum) was first identified for its anti-bacterial activity, and was later also shown to be an efficient anticancer agent. However, the therapeutic use of this anticancer drug is somewhat limited by its toxic side effects, which include nephrotoxicity, nausea, and vomiting. Furthermore the development of drug-resistant tumours is commonly observed following therapy with cisplatin. Hence there is a need for improved platinum derived drugs to overcome these limitations.
Aims Apoptosis contributes significantly to the cytotoxic effects of anticancer agents such as cisplatin; therefore in this study the potential anticancer properties of a series of pyrazole palladium(II) and platinum(II) complexes, $[(3,5\text{-}R_2pz)_2PdCl_2]$ {$R = H$ (**1**), $R = Me$ (**2**)} and $[(3,5\text{-}R_2pz)_2PtCl_2]$ {$R = H$ (**3**), $R = Me$ (**4**)}, were evaluated by assessment of their pro-apoptotic activity.
Methods The induction of apoptosis was measured in CHO cells by the detection of phosphatidylserine (PS) exposure using the annexin V and APOP*ercentage*™ assays; DNA fragmentation using the Terminal deoxynucleotide transferase dUTP Nick End Labelling (TUNEL) assay; and the detection of activated caspase-3.
Results The platinum complexes were shown to be considerably more active than the palladium complexes, with complex **3** demonstrating the highest level of cytotoxic and pro-apoptotic activity. The LD_{50} values for complex **3** and cisplatin were 20 and 70 μM, respectively, demonstrating that the cytotoxic activity for complex **3** was three times higher than for cisplatin. Various human cancer cell lines, including CaSki, HeLa, as well as the p53 mutant Jurkat T cell line were also shown to be susceptible to complex **3**.
Conclusions Collectively, this in vitro study provides insights into action of palladium and platinum complexes and demonstrates the potential use of these compounds, and in particular complex **3**, in the development of new anticancer agents.

Keywords Bis(pyrazole)palladium(II) · Bis(pyrazole)platinum(II) · Cisplatin · Apoptosis · Cytotoxicity · Anticancer

F. K. Keter · J. Darkwa
Department of Chemistry, University of Johannesburg, P.O. Box 524, Auckland Park 2006, South Africa

S. Kanyanda · D. J. G. Rees · M. Meyer (✉)
Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville, Cape Town 7535, South Africa
e-mail: mmeyer@mail.biotech.uwc.ac.za

S. S. L. Lyantagaye
Department of Molecular Biology and Biotechnology, University of Dar es Salaam, P.O. Box 35179, Dar es Salaam, Tanzania

Introduction

The World Health Organization reported that in 1997, a worldwide total of 6.2 million deaths were due to cancer and that the global cancer rate could increase by 50% to 15 million new cases by 2020 [14]. As the prognosis for treatment of many forms of cancer remains poor, there is a need for more efficient drugs to be developed. The discovery of cisplatin as an anticancer drug [25, 33, 34] has provided the basis for the design of a wider range of related compounds with improved therapeutic profiles. Generally because the metal centres of metallo-organic drugs such as cisplatin are positively charged they are able to bind

negatively charged bio-molecules, and thus proteins and nucleic acids are excellent targets for these drugs [20]. However, it has been shown that the reactivity of these compounds is dependent on the stereochemistry of the ligands attached to the metal ion. Thus any novel metal-based anticancer drug should maintain the chemical structure offered by cisplatin and its analogues, while varying the ligands in order to improve bioactivity.

Cisplatin is used extensively in cancer chemotherapy, for example, testicular and ovarian cancer can be very effectively treated with cisplatin and the overall cure rate for testicular cancer treated with cisplatin exceeds 95% [12]. Cisplatin is also widely used to treat various other forms of cancer, including head and neck, cervical and non-small-cell lung cancer [11]. Nucleophilic groups containing oxygen, nitrogen and sulphur atoms with unpaired electrons are able to bind to cisplatin [22]. Since these groups are present in DNA, RNA and proteins, cisplatin is able to form complexes with these biological molecules. There is now a fairly detailed understanding of how metal complexes such as cisplatin bind to DNA and how the DNA is structurally modified as a result of this interaction [1]. The formation of cisplatin–DNA adducts blocks DNA transcription and replication causing cells to undergo G2 cell-cycle arrest [2, 9]. Under normal circumstances the nucleotide excision repair pathway [3] repairs DNA damage [29, 43, 44]. However, DNA damage caused by cisplatin adducts is very poorly repaired in cells, since cisplatin-induced intra-strand cross-links serve as binding targets for high-mobility-group (HMG) proteins because of structural similarity between these intra-strand cross-links and the natural binding sites for HMG proteins [45]. The binding of HMG proteins to cisplatin–DNA adducts causes distortion in DNA structure that inhibits or slows down DNA repair causing G2 cell-cycle arrest [2, 9, 21, 44]. DNA damage functions as a pro-apoptotic stimulus through the activation of p53 dependent apoptotic pathways [18]. The tumour-suppressor protein p53 plays an important role in regulating cell survival in response to DNA damage [22]. It is known that cancer cells that are deficient in wild-type p53 are more susceptible to cisplatin-induced apoptosis [4, 15]. However, it is also known that cisplatin can kill cancer cells in a p53-independent manner [42].

In general, the disruption of regulation of apoptosis plays a significant role in the transformation of normal cells into tumour cells and many genes that regulate apoptosis can be mutated in cancer cells [32]. For example, genes coding for cysteine aspartic acid-specific proteases (caspases) are frequently mutated in cancer cells [24, 26, 37, 38, 40]. It is therefore not surprising that many traditional anticancer chemotherapy drugs exert their cytotoxic effects by indirectly inducing apoptosis, most often as a result of DNA damage [23, 30]. However, a major obstacle in chemotherapy is the indiscriminate killing of both tumour and normal cells. As a result, there is a need to develop improved anticancer drugs that selectively target tumour cells and specifically target the apoptosis pathways.

In spite of cisplatin's considerable use in cancer therapy, there are problems associated with intrinsic and acquired resistance to this drug [39]. Furthermore, side effects that include nephrotoxicity and neurotoxicity limit the usefulness of this drug [41]. Nevertheless, platinum complexes provide one of the more promising classes of novel anticancer drugs that can be used to treat cancer.

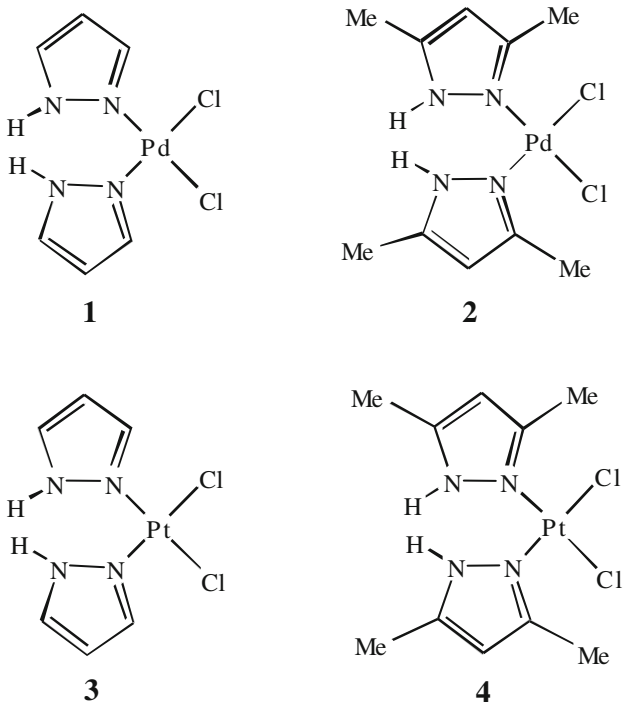
This paper reports the study of the pro-apoptotic activity of palladium and platinum compounds in Chinese hamster ovary (CHO) cells, human cervical epitheloid carcinoma cells (HeLa and CaSki), and human T-cell leukaemia cells (Jurkat). Apoptosis involves a number of biochemical pathways and as a result of a variety of genetic mutations it is known that cancer cells can be selectively resistant to inducers of apoptosis. Since the aim of this study was to evaluate the pro-apoptotic activity of palladium and platinum compounds, we used a normal cell line (CHO) to perform the initial screening for the induction of apoptosis. The characteristic hallmarks of apoptosis (DNA fragmentation, caspase-3 activation, and PS externalisation) were used to investigate the ability of a selection of platinum and palladium-based compounds to activate apoptosis in CHO and several human cancer cell lines. Two palladium compounds (complexes **1** and **2**) and two platinum compounds (complexes **3** and **4**) were evaluated in this study. The platinum complexes were more cytotoxic than the palladium complexes. We demonstrated that the cytotoxicity of complexes **2**, **3**, **4** and cisplatin was due to apoptosis and that complex **3** has the highest pro-apoptotic activity.

It is known that apoptosis contributes to the cytotoxic effects of anticancer drugs. For this reason, compounds with pro-apoptotic activity should be considered as promising candidates for anticancer drug development. Our study demonstrated that the platinum compound, complex **3** shows potential for further drug development.

Materials and methods

Palladium and platinum complexes

Dichloro-bis(pyrazole)palladium(II) (**1**), dichloro-bis(3,5-dimethylpyrazole)palladium(II) (**2**), dichloro-bis(pyrazole) platinum(II) (**3**) and dichloro-bis(3,5-dimethylpyrazole) platinum(II) (**4**) (Scheme 1) were synthesized according to published procedures with minor modifications [28, 36]. The purity of complexes **1**–**4** was established by microanalysis. The following data were obtained for complexes **1**–**4**; **1**(Calc. for $C_6H_8Cl_2N_4Pd$, C, 22.99; H, 2.57; N, 17.87%. Found: C, 23.39; H, 2.25; N, 17.78%); **2** (Calc. for



Scheme 1 Palladium and platinum complexes tested for their anticancer activity. dichloro-bis(pyrazole)palladium(II) (**1**), dichloro-bis(3,5-dimethylpyrazole)palladium(II) (**2**), dichloro-bis(pyrazole) platinum(II), (**3**) and dichloro-bis(3,5-dimethylpyrazole)platinum(II) (**4**)

$C_{10}H_{16}Cl_2N_4Pd$, C, 32.50; H, 4.36; N, 15.16%. Found: C, 32.87; H, 4.50; N, 15.26%); **3** (Calc. for $C_6H_8Cl_2N_4Pt$, C, 17.92; H, 2.01; N, 13.93%. Found: C, 18.31; H, 1.34; N, 13.84%); **4** (Cal. for $C_{10}H_{16}Cl_2N_4Pt$, C, 26.20; H, 3.52; N, 12.23%. Found: C, 26.55; H, 3.16; N, 12.13%). *Cis*-diamminedichloroplatinum(II) (cisplatin) was purchased from Sigma Aldrich.

Cell culture and drug treatment

CHO cells were cultured in Hams F-12 medium containing 1 mM L-glutamine, 5% (v/v) foetal calf serum and 0.2% (v/v) streptomycin–penicillin. HeLa, and CaSki cells were cultured in DMEM medium with GlutaMAX-1, 10% (v/v) foetal calf serum, and 0.2% (v/v) streptomycin–penicillin. Jurkat cells were cultured in RPMI medium with GlutaMAX-1, 10% (v/v) foetal calf serum, and 0.2% (v/v) streptomycin–penicillin. All cell culture reagents were supplied by Invitrogen Ltd. All cell lines were maintained at 37°C in an atmosphere of 5% CO_2. Cells were plated in 6-well tissue culture plates at a cell density of 2.5×10^5 cells per well or in 96-well tissue culture plates at a density of 1×10^4 cells per well. After 24 h, the medium was replaced with medium containing the test compounds. The cells were treated for the indicated times, after which the cells were harvested and the extent of apoptosis was assessed.

Neutral red assay

The effect of the palladium and platinum compounds on the cell viability of CHO cells was determined using the neutral red assay [7]. The cells were plated in 96-well plates at a density of 1×10^4 cells per well and treated with the palladium and platinum complexes. All treatments were performed in triplicate. After 24 h the medium was replaced with medium containing neutral red (16 μg/ml) and the cells were incubated for 3 h. The cells were washed with phosphate buffered saline (PBS) and lysed with an ethanol:acetic acid:water (50%:1%:49%) solution. The concentration of accumulated neutral red, as a marker for cell viability, was measured spectrophotometrically at 560 nm.

APOP*ercentage*™ apoptosis assay

Cells were plated in 96-well tissue culture plates and treated for 24 h with increasing concentrations (20–2,500 μM) of the palladium and platinum compounds. All treatments were performed in triplicate. The cells were washed twice with PBS and stained for 1 h with APOP*ercentage*™ dye (Biocolor Ltd.). Staining was evaluated by light microscopy. The dye uptake was quantified by the colorimetric method according to the manufacturer's instruction. The cells were lysed and the absorbance was measured at 550 nm using a microplate reader.

Annexin V assay

Annexin V (BD Biosciences), a calcium-dependent phospholipid-binding protein with a high affinity for PS, was used to detect and quantify apoptosis. The cells were plated in 6-well tissue culture plates at a cell density of 2.5×10^5 cells per well. The cells were treated for 24 h with cisplatin or complex **3**. The concentrations of cisplatin and complex **3** varied between 100 and 500 μM. Subsequently the cells were removed by trypsinization, washed twice with PBS and stained with annexin-PE and 7-amino-actinomycin D as described in the manufacturer's manual (BD Biosciences). Cell staining was measured by flow cytometry at 575 and 660 nm on a Becton Dickinson FACScan instrument (BD Biosciences). A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences).

Terminal deoxynucleotide transferase dUTP Nick End Labelling (TUNEL) assay

To analyse the occurrence of DNA fragmentation, the TUNEL assay (BD Biosciences) was used [16]. CHO cells were plated in 6-well tissue culture plates at a cell density of 2.5×10^5 cells per well. The cells were treated for 24 h

with complex **3** at concentrations varying between 50 and 200 μM. The cells were removed by trypsinization, washed twice with PBS and fixed for 1 h in 1% paraformaldehyde. The cells were washed twice with PBS and permeabilized for 48 h in 70% ethanol at −20°C. Subsequently the cells were labelled with FITC-dUTP and propidium iodide (PI) as described in the manufacturer's manual (BD Biosciences). Cell staining was measured by flow cytometry at 530 and 585 nm using a Becton Dickinson FACScan instrument (BD Biosciences). A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences). Dual parameter analysis (DNA area signal on the *Y*-axis and DNA width on the *X*-axis) was used to exclude DNA doublet events.

Caspase-3 assay

The activation of caspase-3 was detected using a monoclonal antibody specific for the cleaved caspase-3 (BD Biosciences). CHO cells were plated in 6-well tissue culture plates and treated for 24 h with either cisplatin or complex **3** (500 and 1,000 μM). The cells were removed by trypsinization and washed twice with cold PBS. The cells were resuspended in Cytofix/Cytoperm™. Following 20 min of incubation on ice, the cells were washed twice with Perm/Wash™ buffer and stained for 30 min at room temperature with a FITC-conjugated monoclonal antibody specific for active caspase-3 (BD Biosciences). Cell staining was measured by flow cytometry at 530 nm on a Becton Dickinson FACScan instrument (BD Biosciences). A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences).

Results

Quantitative analysis of cytotoxicity and apoptosis induction

The cytotoxicity of dichloro-bis(pyrazole)palladium(II) (**1**), dichloro-bis(3,5-dimethylpyrazole)palladium(II) (**2**), dichloro-bis(pyrazole)platinum(II), (**3**) and dichloro-bis(3,5-dimethylpyrazole)platinum(II) (**4**) (Scheme 1) was assessed by means of the neutral red assay. CHO cells were treated for 24 h with increasing doses of complexes **1**–**4** and cisplatin.

Cellular viability assay was assessed and used to determine the LD_{50} values for these complexes (Table 1). The LD_{50} values for complexes **1**, **2**, **3**, **4**, and cisplatin were determined to be 10,000, 350, 20, 200, and 70 μM, respectively. Complex **3** displayed the highest cytotoxicity with an LD_{50} value of 20 μM, while complex **1** was the least toxic with an LD_{50} value of 10,000 μM. Shown in Fig. 1b, c, d, e and f are cells treated for 24 h with 2,500 μM of the cisplatin, complexes **1**, **2**, **3**, and **4**, respectively. Figure 1 shows that CHO cells treated with these complexes displayed morphological changes (cell shrinkage and cell detachment) that were indicative of apoptosis. However complex **1** did not induce any morphological changes in the cells at this concentration.

Table 1 LD_{50} of palladium and platinum complexes established in CHO cells. The cells were treated for 24 h with increasing concentrations (20–10 000 μM) of the complexes. Cellular viability was determined using the neutral red assay

Complex	LD_{50} (μM)
1	10,000 ± 0.1
2	350 ± 0.01
3	20 ± 0.01
4	200 ± 0.1
Cisplatin	70 ± 0.01

The APOP*ercentage*™ assay was used to assess whether the cytotoxicity induced by these complexes was the consequence of apoptosis. CHO cells were treated for 24 h with increasing doses (20–2,500 μM) of complexes **1**, **2**, **3**, **4**, and cisplatin, and thereafter stained with the APOP*ercentage*™ dye. The uptake of APOP*ercentage*™ dye was used to evaluate the pro-apoptotic activity of these complexes. Except for complex **1**, all the other complexes including cisplatin caused increased dye-uptake in CHO cells. Complex **1** was unable to induce dye-uptake in these cells at all the concentrations tested. Figure 2 shows that there was a direct relationship between the concentration of the complexes and dye-uptake, that is the absorbance and therefore dye-uptake increased as the concentration of the complexes was increased. Maximum dye-uptake peaked at an absorbance of ∼0.4. For complex **3**, the maximum absorbance was reached at a much lower concentration (500 μM) compared to the other complexes tested.

Comparative analysis of the apoptotic activity of cisplatin and complex 3

The pro-apoptotic activity of complex **3** was further investigated by examining other markers of apoptosis. The annexin V assay, which measures PS externalisation; the caspase-3 assay, which measures caspase-3 cleavage; and the TUNEL assay, which measures DNA fragmentation, were used to perform a comparative analysis of the pro-apoptotic activity of complex **3** and cisplatin. CHO cells were treated for 24 h with increasing doses (100–250 μM) of cisplatin or complex **3**. The level of apoptosis was assessed by means of the annexin V assay and showed no significant difference in the pro-apoptotic activity of these two complexes at low doses (100–120 μM)

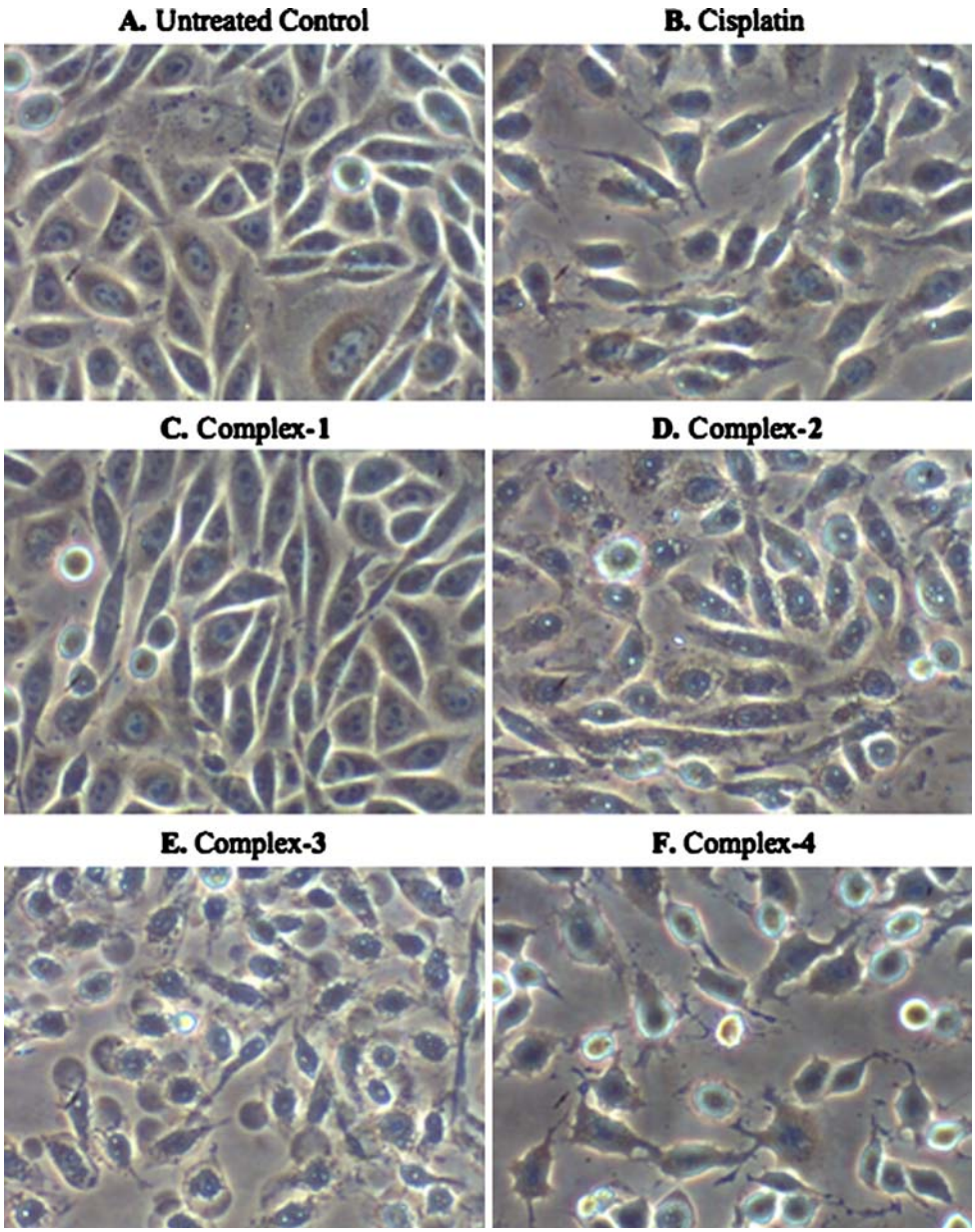


Fig. 1 Palladium and platinum complexes induce morphological changes in CHO cells that are indicative of apoptosis. CHO cells were treated for 24 h with 2,500 μM of these complexes and evaluated by light microscopy. Cells were viewed with a Nikon inverted light microscope at 20× magnification and photographs were taken using a Leica EC3 digital camera. **a** shows untreated cells, while **b**, **c**, **d**, **e** and **f** shows cells treated with *cisplatin*, complexes ***1***, ***2***, ***3***, and ***4***, respectively

as indicated by the number of annexin V positive cells (Fig. 3). The lower left quadrant in the dot plots represents the viable cells, the lower right quadrant represents the early apoptotic cells and the upper right quadrant represents the late apoptotic and necrotic cells. Indicated on these plots is the combined proportion of early and late apoptotic cells. At lower doses (100–120 μM) both complexes induced apoptosis in ~30% of the cells. However, at higher doses (200 and 250 μM), complex **3** was significantly more effective than cisplatin. This is illustrated in Fig. 3, which shows that the number of apoptotic cells for cisplatin and complex **3** at 200 μM were 37 and 85%, respectively. At the highest dose tested (250 μM), the number of apoptotic cells for cisplatin and complex **3** were 40 and 96%, respectively.

To assess the activation of caspase-3, CHO cells were treated for 24 h with 500 μM cisplatin or complex **3** (Fig. 4). The cells were permeabilised and stained for the presence of cleaved caspase-3 using an anti-active caspase-3-PE antibody. Cell fluorescence was measured by FACS analysis. The number of active caspase-3 positive cells for cisplatin- and complex **3**-treated cells was 16 and 86%, respectively (Fig. 4).

DNA fragmentation was evaluated using the TUNEL assay. CHO cells were treated for 24 h with three doses (50, 100, and 200 μM) of complex **3** and subsequently analysed for DNA fragmentation (Fig. 5). As a positive control for this experiment, cells were also treated with 1 μM staurosporine. Fluorescent labelling of fragmented DNA with the TUNEL assay was performed in combination with propidium

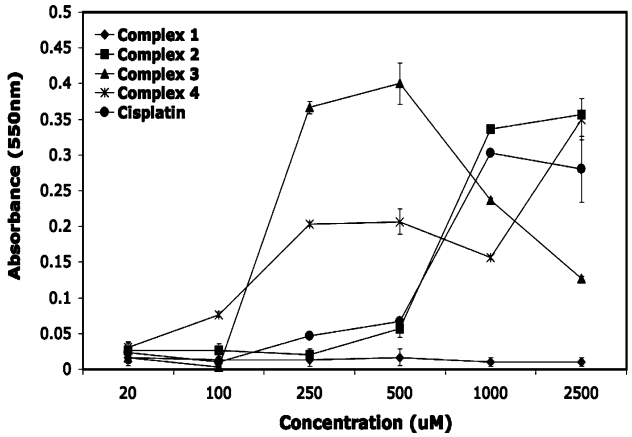


Fig. 2 Quantification of the pro-apoptotic activity of palladium and platinum complexes. CHO cells were treated for 24 h with increasing doses (20–2,500 μM) of complexes ***1***, ***2***, ***3***, ***4***, and *cisplatin*. Apoptosis was measured by the APOP*ercentage*™ assay as described by the manufacturers. The cells were stained with APOP*ercentage*™ dye and dye-uptake, which is a measure of apoptosis, was measured at A_{550}. The *error bars* show ±standard error for each measurement

iodide (PI) counterstaining and cell fluorescence was measured by FACS analysis (Fig. 5). The events in the R2 region of the dot plot represent cells that are negative for FITC-dUTP staining and therefore do not display any DNA fragmentation, while events in the R3 region represent cells that are positive for FITC-dUTP and DNA fragmentation. Figure 5 shows a dose dependent increase from 8 to 29% in the number of cells staining positive for FITC-dUTP incorporation, confirming that complex **3** does induce DNA fragmentation.

Complex **3** induces apoptosis in cancer cells

Various human cancer derived cell lines, including Jurkat, CaSki and HeLa were screened for susceptibility to complex **3**. The cells were treated for 24 h with cisplatin (250 μM) or complex **3** (250 μM). Apoptosis was assessed by the annexin V assay (Fig. 6). The numbers on the dot plots indicate the total number of apoptotic cells, that is, the combined proportion of early and late apoptotic cells. The apoptotic cell death counts for Jurkat, CaSki and HeLa cells treated for 24 h with cisplatin were 92, 45 and 72%, respectively. While the apoptotic cell death counts for cells treated with complex **3** were 95, 65 and 58%, respectively.

Discussion

Four palladium(II) and platinum(II) pyrazole complexes (Scheme 1) were chosen for this study for two reasons. Firstly, they all resemble cisplatin structurally and are expected to bind biological molecules in a similar manner. Secondly, the electronic properties of pyrazole ligands offer metal centres for late transition metals that are quite electrophilic to allow coordination to biological molecules.

The measurement of cellular viability using the supravital dye, neutral red is based on the ability of live cells to incorporate and bind the dye. Neutral red readily penetrates cell membranes and accumulates intracellularly in lysosomes. Physiological conditions that affect lysosomal membrane integrity, such as cytotoxicity for example, result in decreased neutral red uptake and hence it is possible to differentiate viable cells from damaged or dead cells. In this study the neutral red assay was used to determine the LD_{50} values for the palladium and platinum complexes. Based on the LD_{50} values, the platinum compounds (complexes **3** and **4**) were more cytotoxic than the palladium compounds (complexes **1** and **2**) (Table 1). Complex **3** was the most active complex with an LD_{50} value of 20 μM.

Cellular viability assays such as the neutral red assay measure cytotoxicity and therefore do not distinguish between apoptosis and necrosis. The aim of this study was to evaluate the use of these complexes as possible anticancer agents in terms of their ability to induce apoptosis. Hence the complexes were also tested for pro-apoptotic activity using the APOP*ercentage*™ apoptosis assay (Biocolor Ltd.).

At a concentration of 2,500 μM, complexes **2**, **3**, **4** and cisplatin induced morphological changes (cell shrinkage and cell detachment) in the CHO cells that were indicative of cells undergoing apoptosis (Fig. 1). However, no morphological changes were observed in CHO cells treated with complex **1**. The APOP*ercentage*™ apoptosis assay confirmed that cells treated with complexes **2**, **3**, **4** and cisplatin were undergoing apoptosis. The APOP*ercentage*™ apoptosis assay is a dye-uptake bioassay, which detects apoptotic cells during PS trans-membrane movement. PS externalisation is a characteristic feature associated with cells dying via apoptosis, hence only apoptotic cells will take up the dye. This assay allows for the detection and quantification of apoptosis since the trapped dye can be released from the cells and the total dye-uptake can be measured. The APOP*ercentage*™ dye was taken up by CHO cells treated with complexes **2**, **3**, **4** and cisplatin. Quantification of the dye released from these cells shows that there is a direct relationship between the concentration of the complexes and the level of apoptosis (Fig. 2). Higher A_{550} values means that more dye was taken up by the cells and that more cells died via apoptosis. A maximum A_{550} value was reached at 0.4 and complex **3** stands out as the most active complex, since it was capable of inducing maximum APOP*ercentage*™ dye uptake at 500 μM. In comparison, complexes **2**, **4** and cisplatin required concentrations up to five times higher (2,500 μM) to induce similar levels of apoptosis. The A_{550} value for cells treated with complex **1**

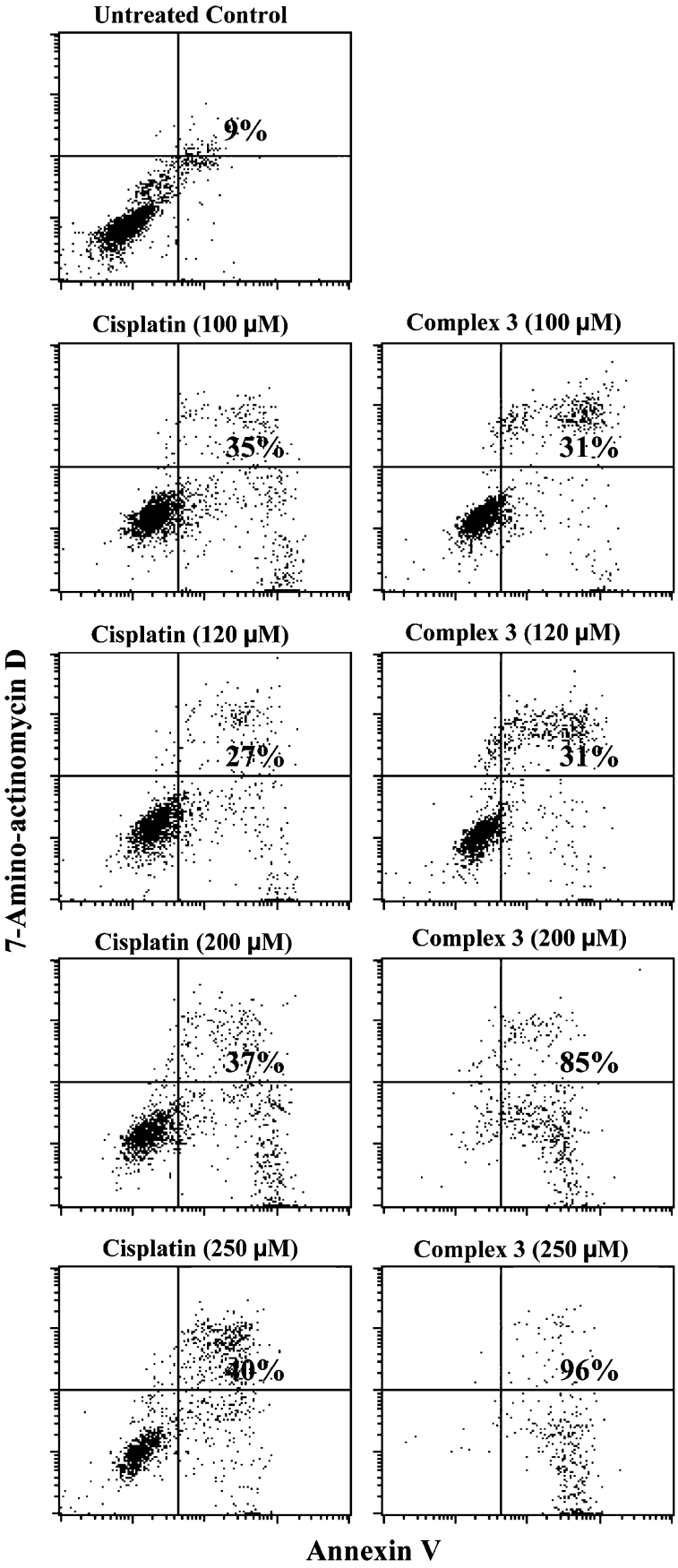


Fig. 3 Comparison of the pro-apoptotic activity of complex **3** and cisplatin. CHO cells were treated for 24 h with increasing doses (100–250 µM) of *complex* 3 or *cisplatin* and evaluated for PS externalization using the annexin V assay. For each dot plot the *lower left quadrant* represents the normal viable cells, the *lower right quadrant* represents the early apoptotic cells and the *upper right quadrant* represents the late apoptotic and necrotic cells. Indicated on the plot in the *upper right quadrant* is the combined early and late apoptotic cell numbers

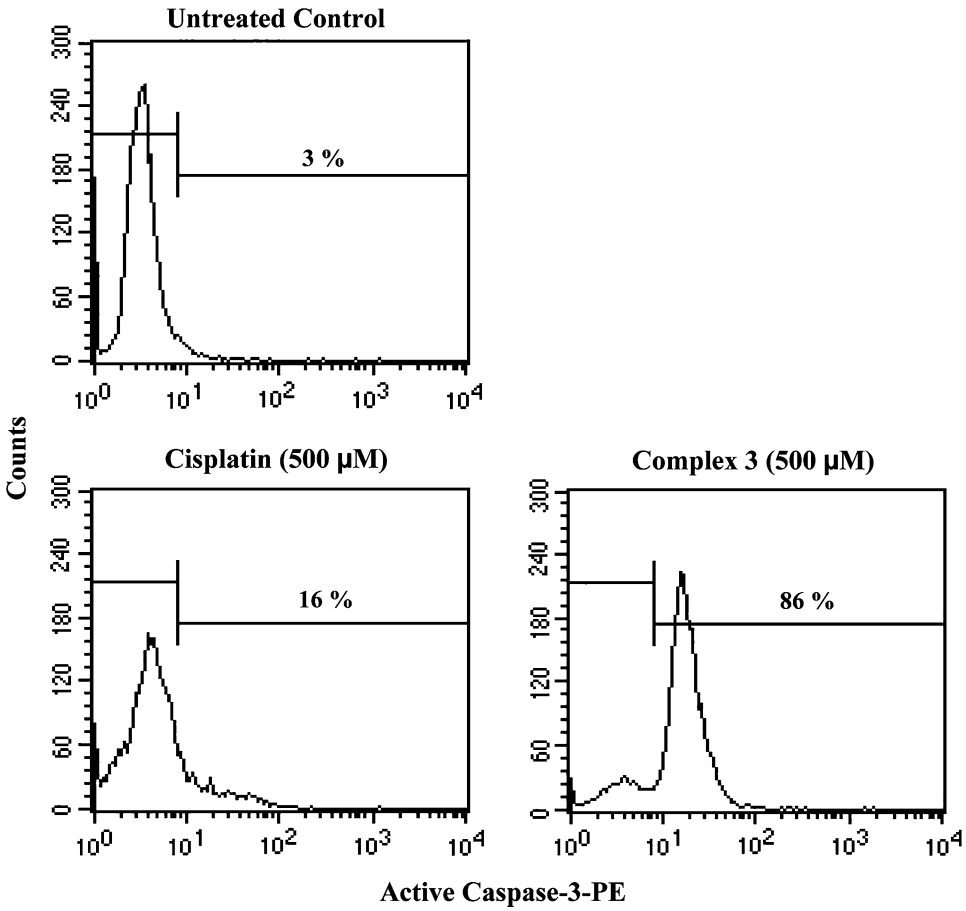


Fig. 4 Evaluation of caspase-3 activation by complex **3** and cisplatin. CHO cells were treated for 24 h with 500 µM of *complex 3* or *cisplatin*. The cells were permeabilised and stained for the presence of active caspase-3 using an anti-active caspase-3-PE antibody. Cell fluorescence was measured by flow cytometry. Indicated on the histogram plots is the number of cells staining positive for the anti-active caspase-3 antibody

remained at the same level as the A_{550} value for the untreated cells, even at concentrations as high as 2,500 µM suggesting that complex **1** did not induce apoptosis. This result is in agreement with the data from the cellular viability assay. Furthermore, the lack of morphological changes observed in cells treated with this complex suggested that complex **1** was not cytotoxic when tested at concentrations below 2,500 µM. Overall, the platinum compounds (complexes **3**, **4** and cisplatin) displayed higher pro-apoptotic activity than the palladium compounds (complexes **1** and **2**). In addition, the pro-apoptotic activity of complexes **3** and **4** were also higher than cisplatin. Based on the APOP*ercentage*™ assay the pro-apoptotic activity of complex **3** is almost eight times higher than for cisplatin.

Despite palladium and platinum being elements of the same group and the ionic radii of these elements being nearly the same as a result of lanthanoid contraction, their respective pyrazole complexes exhibit different activities. We attribute this to an associative substitution mechanism similar to that reported by Rauterkus et al. [31], in which the kinetic behaviour of the two metal complexes differ. Platinum compounds have been reported to be more stable in solution than palladium compounds [10] and thus would be less susceptible to translabilization, especially in the biological milieu, as opposed to palladium compounds. This is substantiated by the fact that both palladium and platinum compounds used in this study have the same ligand system and that the only difference in activities is attributed to the metals; thus the observed activities could only be due to the solution properties and perhaps the intrinsic properties of the metals, where platinum compounds were considerably better compared to palladium compounds. It is also worth noting that the palladium complexes had poor solubility compared to platinum analogues. Platinum compounds are found to be more stable in solution than palladium compounds and thus would be less susceptible to translabilization as opposed to palladium compounds. It is for this reason that their activities are considerably better compared to palladium compounds.

The bulkiness of the pyrazole ligands also appears to affect activity. For example the activity of complex **3** was significantly higher than that of cisplatin. Previous studies also show that the more bulky dichloro-2-(2-pyridyl)benzimidazoleplatinum(II) is more active than cisplatin [17]. The steric hindrance due to 2-(2-pyridyl)benzimidazole ligands was found to reduce rapid detoxification by thiol-containing molecules, as compared to the ammonia ligands in cisplatin. There is a probability that such bulky ligands prevent translabilization and undesired displacement of the non-leaving ligand by other nitrogen donors [17]. Hence it

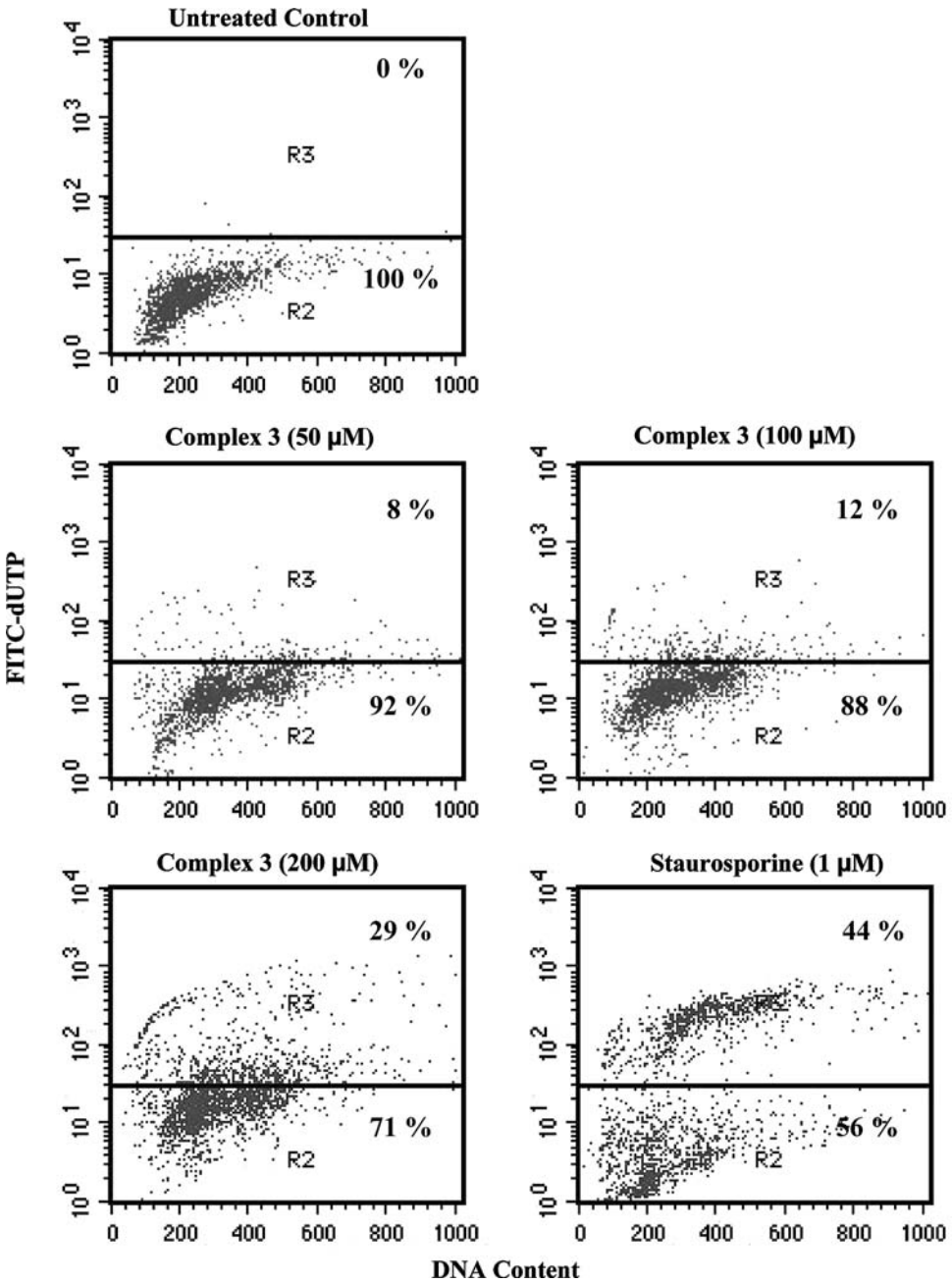


Fig. 5 Complex **3** induces DNA fragmentation in CHO cells. CHO cells were treated for 24 h with increasing doses (50–200 μM) of complex **3**. As a positive control, cells were treated with 1 μM staurosporine, DNA fragmentation was assessed by the TUNEL assay using FITC-dUTP to label the apoptotic cells. Cell fluorescence was measured by flow cytometry. Cells within the *R2* region represents the normal viable cells with intact genomic DNA while cells within the *R3* region are apoptotic cells with fragmented DNA which stain positive for FITC-dUTP. Indicated on the dot plots are the cell numbers in the *R2* and *R3* regions

is possible that complex **3** could be acting through a similar mechanism that induces the formation of DNA-adducts, promoting the activation of apoptosis. Earlier structure–activity relationships have defined the necessity of at least one NH moiety, which is thought to be important for hydrogen bonding interactions to DNA [6]. It has been suggested that the small size of the NH group, rather than its hydrogen bonding ability, is responsible for the greater activity exhibited by platinum compounds with amine carrier ligands bearing multiple NH protons [8]. However, it should be noted that if the carrier ligands are sterically crowded, then the presence of NH group(s) is not necessary. Based on these observations, we propose that the complexes reported in this paper which also have NH moieties, act in the same manner, that is, the non-covalent interaction of the NH moieties of complexes **1**–**4** with DNA, led to a kinked platinated-DNA adduct, which played a crucial role in the activities observed.

Since the APOP*ercentage*™ assay demonstrated higher activity for complex **3**, the other compounds (complexes **1**, **2**, and **4**) were excluded from further analysis. In addition, both the neutral red assay and the APOP*ercentage*™ assay demonstrated that complex **3** was a more efficient cytotoxic agent than cisplatin. We therefore further evaluated the pro-apoptotic activity of complex **3** in comparison to cisplatin. Similarly to the

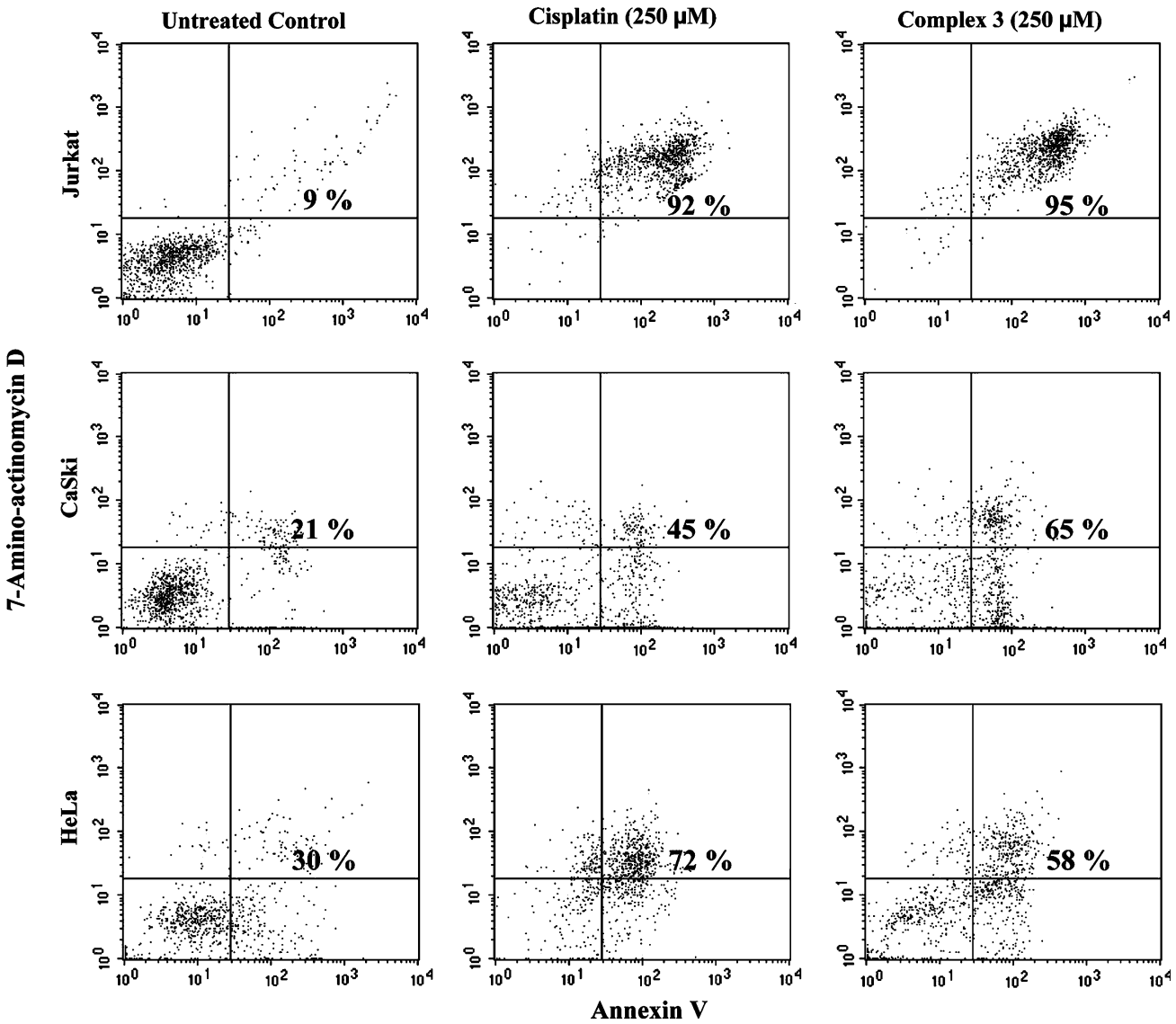


Fig. 6 Evaluation of pro-apoptotic activity of complex **3** in human cancer cell lines. Various cancer cell lines including *Jurkat*, *HeLa*, and *CaSki* were treated for 24 h with *complex 3* (250 µM) or *cisplatin* (250 µM). Apoptosis was assessed by the annexin V assay. For each dot plot, the *lower left quadrant* represents the normal viable cells, the *lower right quadrant* represents the early apoptotic cells and the *upper right quadrant* represents the late apoptotic and necrotic cells. Indicated on the plot in the *upper right quadrant* is the combined early and late apoptotic cell numbers

APOP*ercentage*™ assay, the annexin V assay also detects apoptosis during the PS trans-membrane movement phase. An added advantage is the sensitivity of this assay at detecting the early membrane changes during apoptosis [5]. Both cisplatin and complex **3** induced PS externalisation in CHO cells after 24 h as measured by the annexin V assay (Fig. 3). However, complex **3** was significantly more bioactive than cisplatin at doses above 200 µM. All three assays (neutral red, APOP*ercentage*™ and annexin V) used in this study demonstrated that the cytotoxicity of complex **3** is much higher than for cisplatin.

Several nucleases have been associated with DNA fragmentation during apoptosis. Among these nucleases is the Caspase Activated Deoxyribonuclease (CAD), which pre-exists in living cells as an inactive complex with an inhibitory sub-unit, called ICAD. The cleavage of CAD/ICAD complex, that is activation of CAD, occurs by means of caspase-3 mediated cleavage of the inhibitory sub-unit (ICAD) [35]. It is well known that cisplatin-induced apoptosis can occur via caspase-3 activation [19]. However, it has also been shown that cisplatin-induced apoptosis in A2780 ovarian tumour cells occurs via a caspase-3 independent pathway [19]. Hence we also investigated whether the pro-apoptotic activity of complex **3** involved the activation of caspase-3. Both cisplatin and complex **3** induced caspase-3 cleavage in CHO cells. However, as shown in Fig. 4, the

extent of caspase-3 cleavage in cells treated with complex **3** was ~5 fold higher than that in cells treated with cisplatin.

Farrell et al. [13] previously demonstrated that the pro-apoptotic activity of platinum compounds is associated with the production of DNA strand breaks. Hence we also investigated the ability of complex **3** to induce DNA fragmentation. CHO cells were treated with increasing concentrations (50–200 μM) of complex **3** and evaluated for the presence of fragmented DNA using the FITC-dUTP and the TUNEL assay. Figure 5 shows a dose-dependent increase (7–29%) in the percentage of cells staining positive for fragmented DNA.

We investigated the ability of complex **3** to induce apoptosis in various cancer derived cell lines, which included Jurkat, CaSki and HeLa. Jurkat cells were more sensitive to the effects of both cisplatin and complex **3**, with ~90% of the cells undergoing apoptosis when treated with 250 μM of the complexes (Fig. 6). The human cervical epitheloid carcinoma cells (CaSki and HeLa) were more resistant to these platinum compounds. CaSki and HeLa cells were differentially susceptible to cisplatin and complex **3**. CaSki cells appeared to be more sensitive to complex **3**, whereas the HeLa cells were more susceptible to cisplatin. This may point to different mechanistic actions for these two complexes. Alternatively this differential susceptibility may be a reflection of different genetic mutations that block apoptotic pathways in these cell lines. Mutations in genes regulating apoptosis have been reported for many human cancer cell lines. For example, point mutations in the p53 gene prevent the expression of this oncogene in Jurkat T cells [27]. The susceptibility of cancer cells to these complexes highlights the possible use of complex **3** as a potential anticancer agent.

The results obtained from this study demonstrate that the metal complexes evaluated have significant cytotoxic activity and that the cell death induced by these complexes is due to the activation of apoptosis. Various markers of apoptosis, including PS externalisation, caspase-3 activation and DNA fragmentation were detected in cells treated with these metal compounds. This study also shows that the platinum complexes are considerably more active than the palladium complexes and that one of the platinum complexes [dichlorobis(pyrazole)platinum(II) or complex **3**] is also active against various cancer cells including Jurkat, CaSki and HeLa. Furthermore this study also shows that complex **3** is significantly more active than cisplatin and represents a useful point of departure for future chemical diversification of this group of compounds to increase their activity and solubility.

References

1. Admiraal G, Van der Veer JL, Rudolf DAG, Jeroen HJD, Reedijk J (1987) Intrastrand Bis(guanine) Chelation of d(CpGpG) to cis -Platinum: an X-ray single-crystal structure analysis. J Am Chem Soc 109:592–594
2. Allday MJ, Inman GJ, Crawford DH, Farrell PJ (1995) DNA damage in human B cells can induce apoptosis, proceeding from G1/S when p53 is transactivation competent and G2/M when it is transactivation defective. EMBO J 14:4994–5005
3. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A (2004) Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 350:2343–2351
4. Anthoney DA, McIlwrath AJ, Gallagher WM, Edlin AR, Brown R (1996) Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. Cancer Res 56:1374–1381
5. Anthony RS, McKelvie ND, Cunningham AJ, Craig JI, Rogers SY, Parker AC (1998) Flow cytometry using annexin V can detect early apoptosis in peripheral blood stem cell harvests from patients with leukaemia and lymphoma. Bone Marrow Transplant 21:441–446
6. Bloemink MJ, Engelking H, Karentzopoulos S, Krebs B, Reedijk J (1996) Synthesis, crystal structure, antitumor activity, and DNA-binding properties of the new active platinum compound (bis(*N*-methylimidazol-2-yl)carbinol)dichloroplatinum(II), lacking a NH moiety, and of the inactive analog dichloro(N1,N1′-dimethyl-2,2′-biimidazole)platinum(II). Inorg Chem 35:619–627
7. Borenfreund E, Borrero O (1984) In vitro cytotoxicity assays. Potential alternatives to the Draize ocular allergy test. Cell Biol Toxicol 1:55–65
8. Carlone M, Marzilli LG, Natile G (2004) Platinum complexes with NH groups on the carrier ligand and with only one guanine or hypoxanthine derivative. Informative models for assessing relative nucleobase and nucleotide hydrogen-bond interactions with amine ligands in solution. Inorg Chem 43:584–592
9. Chu G (1994) Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair. J Biol Chem 269:787–790
10. Cotton FA, Wilkinson G (1972) Advanced inorganic chemistry. Wiley & Sons, New York
11. Cvitkovic E, Misset JL (1996) Chemotherapy for ovarian cancer. N Engl J Med 334:1269, author reply 1270
12. Einhorn EH (1997) Testicular cancer: an oncological success story. Clin Cancer Res 3:2630–2632
13. Farrell N, Povirk LF, Dange Y, DeMasters G, Gupta MS, Kohlhagen G, Khan QA, Pommier Y, Gewirtz DA (2004) Cytotoxicity, DNA strand breakage and DNA-protein crosslinking by a novel transplatinum compound in human A2780 ovarian and MCF-7 breast carcinoma cells. Biochem Pharmacol 68:857–866
14. Frankish H (2003) 15 million new cancer cases per year by 2020, says WHO. Lancet 361:1278
15. Gallagher WM, Cairney M, Schott B, Roninson IB, Brown R (1997) Identification of p53 genetic suppressor elements which confer resistance to cisplatin. Oncogene 14:185–193
16. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
17. Gumus F, Algul O, Eren G, Eroglu H, Diril N, Gur S, Ozkul A (2003) Synthesis, cytotoxic activity on MCF-7 cell line and mutagenic activity of platinum(II) complexes with 2-substituted benzimidazole ligands. Eur J Med Chem 38:473–480
18. Henkels KM, Turchi JJ (1997) Induction of apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cell lines. Cancer Res 57:4488–4492
19. Henkels KM, Turchi JJ (1999) Cisplatin-induced apoptosis proceeds by caspase-3-dependent and -independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. Cancer Res 59:3077–3083
20. Holford J, Sharp SY, Murrer BA, Abrams M, Kelland LR (1998) In vitro circumvention of cisplatin resistance by the novel

sterically hindered platinum complex AMD473. Br J Cancer 77:366–373
21. Huang JC, Zamble DB, Reardon JT, Lippard SJ, Sancar A (1994) HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. Proc Natl Acad Sci USA 91:10394–10398
22. Jordan P, Carmo-Fonseca M (2000) Molecular mechanisms involved in cisplatin cytotoxicity. Cell Mol Life Sci 57:1229–1235
23. Kaufmann SH, Earnshaw WC (2000) Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256:42–49
24. Kim HS, Lee JW, Soung YH, Park WS, Kim SY, Lee JH, Park JY, Cho YG, Kim CJ, Jeong SW, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH (2003) Inactivating mutations of caspase-8 gene in colorectal carcinomas. Gastroenterology 125:708–715
25. Kociba RJ, Sleight SD, Rosenberg B (1970) Inhibition of Dunning asc itic leukemia and Walker 256 carcinosarcoma with *cis*-diamminedichloroplatinum (NSC-119875). Cancer Chemother Rep 54:325–328
26. Kurokawa H, Nishio K, Fukumoto H, Tomonari A, Suzuki T, Saijo N (1999) Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells. Oncol Rep 6:33–37
27. Laumann R, Jucker M, Tesch H (1992) Point mutations in the conserved regions of the p53 tumour suppressor gene do not account for the transforming process in the Jurkat acute lymphoblastic leukemia T-cells. Leukemia 6:227–228
28. Li K, Darkwa J, Guzei IA, Mapolie SF (2002) Synthesis and evaluation of substituted pyrazoles palladium(II) complexes as ethylene polymerization catalysts J. Organomet Chem 660:108–115
29. Lindahl T, Karran P, Wood RD (1997) DNA excision repair pathways. Curr Opin Genet Dev 7:158–169
30. Lowe SW, Lin AW (2000) Apoptosis in cancer. Carcinogenesis 21:485–495
31. Rauterkus MJ, Fakih S, Mock C, Puscasu I, Krebs B (2003) Cisplatin analogues with 2,2′-dipyridylamine ligands and their reactions with DNA model nucleobases Inorg. Chim Acta 350:355–365
32. Renehan AG, Booth C, Potten CS (2001) What is apoptosis, and why is it important? BMJ 322:1536–1538
33. Rosenberg B, VanCamp L, Trosko JE, Mansour VH (1969) Platinum compounds: a new class of potent antitumour agents. Nature 222:385–386
34. Rossof AH, Slayton RE, Perlia CP (1972) Preliminary clinical experience with cis-diamminedichloroplatinum (II) (NSC 119875, CACP). Cancer 30:1451–1456
35. Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99
36. Sakai K, Tomita Y, Ue T, Goshima K, Ohminato M, Tsubomura T, Matsumoto K, Ohmura K, Kawakami K (2000) Syntheses, antitumor activity, and molecular mechanics studies of *cis*-PtCl2(pzH)2 (pzH = pyrazole) and related complexes. Crystal structure of a novel magnus-type double-salt [Pt(pzH)4][PtCl4][*cis*-PtCl2(pzH)2]2 involving two perpendicularly aligned 1D chains. Inorg Chim Acta 297:64–71
37. Schwartz S Jr, Yamamoto H, Navarro M, Maestro M, Reventos J, Perucho M (1999) Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype. Cancer Res 59:2995–3002
38. Shin MS, Kim HS, Kang CS, Park WS, Kim SY, Lee SN, Lee JH, Park JY, Jang JJ, Kim CW, Kim SH, Lee JY, Yoo NJ, Lee SH (2002) Inactivating mutations of CASP10 gene in non-Hodgkin lymphomas. Blood 99:4094–4099
39. Siddik ZH (2003) Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 22:7265–7279
40. Soung YH, Lee JW, Kim HS, Park WS, Kim SY, Lee JH, Park JY, Cho YG, Kim CJ, Park YG, Nam SW, Jeong SW, Kim SH, Lee JY, Yoo NJ, Lee SH (2003) Inactivating mutations of CASPASE-7 gene in human cancers. Oncogene 22:8048–8052
41. Wang D, Lippard SJ (2005) Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 4:307–320
42. Zamble DB, Jacks T, Lippard SJ (1998) p53-Dependent and -independent responses to cisplatin in mouse testicular teratocarcinoma cells. Proc Natl Acad Sci USA 95:6163–6168
43. Zamble DB, Lippard SJ (1995) Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem Sci 20:435–439
44. Zamble DB, Mu D, Reardon JT, Sancar A, Lippard SJ (1996) Repair of cisplatin–DNA adducts by the mammalian excision nuclease. Biochemistry 35:10004–10013
45. Zlatanova J, Yaneva J, Leuba SH (1998) Proteins that specifically recognize cisplatin-damaged DNA: a clue to anticancer activity of cisplatin. FASEB J 12:791–799