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Novel Antitumor Cisplatin and Transplatin Derivatives Containing 1-Methyl-7-Azaindole: Synthesis, Characterization and Cellular Responses

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ABSTRACT: The current work investigates the effect of new bifunctional and mononuclear Pt(II) compounds, the cis- and trans-isomers of $[PtCl_2(NH_3)(L)]$ (L = 1-methyl-7-azaindole, compounds **1** and **2**, respectively), on growth and viability of human carcinoma cells as well as their putative mechanism(s) of cytotoxicity. The results show that substitution of 1-methyl-7-azaindole for ammine in cisplatin or transplatin results in an increase of the toxic efficiency, selectivity for tumor cells in cisplatin-resistant cancer cells and activation of the trans geometry. The differences in the cytotoxic activities of **1** and **2** were suggested to be due to their different DNA binding mode, different capability to induce cell cycle perturbations and fundamentally different role of transcription factor p53 in their mechanism of action. Interestingly, both isomers make it possible to detect their cellular uptake and distribution in living cells by confocal microscopy without their modification with an optically active tag.

INTRODUCTION

Cisplatin [*cis*-diamminedichloridoplatinum(II)] and other platinum-based anticancer agents are well known to be effective in the treatment of many human malignancies. However, the ultimate success of these agents is often limited by the natural or acquired drug resistance and side effects. These drawbacks have fostered the search for new antitumor platinum-based drugs with fewer side effects and more effective outcomes. New mononuclear bifunctional platinum drugs have been derived from cisplatin or ineffective transplatin [*trans*-diamminedichloridoplatinum(II)] by replacing one or both ammine groups with other nonleaving N-donor ligands. The design of such derivatives has been inspired by the observation that the carrier ligands can deeply affect the pharmacological behavior of the drug including cell accumulation and distribution, activation, DNA binding, and cellular responses to the DNA damage. In other words, nonleaving ligands of cisplatin or transplatin derivatives can modulate their biological properties.[1-6]

Some of us have shown in recent papers[7, 8] that replacement of the ammine ligands of cisplatin by 7-azaindole derivatives results in considerable enhancement of cytotoxicity against several human cancer cell lines. Thus, the rational chemical design can be applied also to this class of compounds since key substituents at the 7-azaindole ring system can play a major role in controlling the chemical and biological properties, such as cellular accumulation and distribution, effects at the level of cell cycle regulation, binding to DNA, propensity for DNA adduct repair, and reactivity toward sulfur-containing nucleophiles. Hence, substituted 7-azaindoles can offer a good opportunity for the synthesis of novel therapeutic agents.

The rational design of novel platinum anticancer drugs can also benefit from early information on the trafficking and localization of platinum drugs in tumor cells. Therefore, confocal microscopy has been successfully applied to investigate the cellular distribution of Pt

complexes. However, this relatively simple detection technique, which enables the visualization of compounds in living cells, requires the use of drugs which, after illumination, emit radiation detectable with sufficiently sensitive detectors. Cisplatin and most Pt anticancer complexes do not possess this property and need to be modified with an optically active tag. These alterations can affect chemical properties of the complexes, creating artifacts such as altered specificity and other biological effects. Hence, in order to obtain significant information about the *in vivo* trafficking and localization of a platinum drug in living tumor cells, the modified platinum complex must be as similar as possible to the real drug.

Several attempts have been made to obtain high-resolution images of the cellular accumulation and subcellular distribution of fluorescein-tagged Pt(II) complexes (see Refs.[9-11] as examples). Interestingly, derivatives of cisplatin and transplatin with one ammine and one aromatic N-donor heterocycle (L) (*cis*- and *trans*-[$PtCl_2(NH_3)L$]) have proved to be endowed with good antitumor activity and one of these compounds, picoplatin [*cis*-amminedichlorido(2-methylpyridine)platinum(II)], is under advanced clinical trials with changing fortunes.[12, 13] This opened the possibility to use a N-donor heterocycle which is intrinsically fluorescent, such as 7-azaindole,[14] for monitoring the cell permeation and distribution of the platinum drugs. However, coordination to platinum results in almost complete quenching of the fluorescence. In addition, 7-azaindole exhibits a pH-dependent fluorescence. Hence, since the pH is different in different cellular compartments, the use of a probe, whose fluorescence is dependent on pH, appears disadvantageous. The latter drawback can be circumvented by derivatization of 7-azaindole with a methyl group.

In the present work we investigate the cytotoxic potential and molecular and cellular pharmacology of a new cisplatin derivative bearing a nonleaving 1-methyl-7-azaindole ligand (hereafter called 1M7AI) and its trans isomer. We report the synthesis and characterization of

both the cis- and trans-isomers of $[PtCl_2(NH_3)(1M7AI)]$ (**1** and **2**, respectively, Figure 1), the crystal structure of **1**, and several biological properties of **1** and **2**, such as cytotoxicity, cellular accumulation, impact on cell cycle regulation, triggering of apoptosis and involvement of p53 pathway in the mechanism of action. Moreover, the optical properties were utilized to follow the cellular internalization and distribution of the complexes in tumor cells.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Platinum Complexes. The Pt(II) complexes under investigation have one ammine and one N-donor aromatic heterocycle (1M7AI). For comparison, both the *cis*- and *trans*-$[PtCl_2(NH_3)(1M7AI)]$ isomers were synthesized and fully characterized.

The cis complex, *cis*-$[PtCl_2(NH_3)(1M7AI)]$ (**1**), was prepared according to a previously reported procedure for the synthesis of analogous compounds,[14, 15] with some modifications. Starting from $K[PtCl_3NH_3]$,[12] the reaction with 1M7AI leads to substitution of one of the two trans chlorides (both cis to the ammine) which are mutually trans-labilized. The reaction is quite slow at room temperature (complete in 4 days) and affords the desired product in moderate yield, the main side-product being the cationic species $[PtCl(NH_3)(1M7AI)_2]Cl$ that can be easily removed (Scheme S1).

The synthesis of the trans complex, *trans*-$[PtCl_2(NH_3)(1M7AI)]$ (**2**), requires a different procedure in which *cis*-$[PtI_2(NH_3)_2]$ is first converted to *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ (reaction with $AgNO_3$) and then to *cis*-$[Pt(NH_3)_2(1M7AI)_2]^{2+}$ (reaction with 1M7AI. 1:2 ratio). Treatment with excess KI – a ligand with strong trans-labilizing effect – leads to substitution of two trans ligands (one ammine and one 1M7AI) and formation of *trans*-$[PtI_2(NH_3)(1M7AI)]$. The diiodido species

can be converted to the dichlorido species **(2**) by reaction with $AgNO_3$ (1:2 molar ratio) in water solution followed by addition of excess KCl (Scheme S2).

Both **1** and **2** were characterized by elemental analysis, ESI-MS, and ^{1}H and ^{195}Pt NMR spectroscopy. The ^{1}H-spectra of both compounds show five signals between 9 and 6.5 ppm belonging to aromatic protons, one singlet close to 5.2 ppm belonging to the methyl group, and one broad signal close to 4.0 ppm belonging to the amminic protons. In general, protons are more shielded in the *trans* than in the *cis* isomer. It is noteworthy the remarkable downfield shift ($\Delta\delta >$ 1.30 ppm) of the methyl protons upon coordination of 1M7AI to platinum which witnesses the closeness of the methyl group to the deshielding cone originated by the platinum quadrupole in the direction perpendicular to the coordination plane. The assignment of individual aromatic protons was accomplished by 2D COSY experiments (Figure S1, see Schemes S1 and S2 for the numbering of protons).

The coordination of 1M7AI to platinum via N7 causes a $^{3}J_{H6,Pt}$ coupling which is greater for **1** (1M7AI *trans* to Cl, $^{3}J_{H6,Pt} = 41$ Hz) than for **2** (1M7AI *trans* to NH_3, $^{3}J_{H6,Pt} = 32$ Hz) indicating that the ligand trans to 1M7AI has a better σ-donor ability in **2** (NH_3) than in **1** (Cl^-). These results are in full agreement with literature data,[16] and fully support the assignment of the isomer configurations (*cis* or *trans*). The ^{195}Pt chemical shifts were nearly coincident for **1** and **2** (–1,970 and –1,967 ppm, respectively) and fall in the range found for analogous compounds with N_2Cl_2 set of donor atoms.[17] It is interesting to note that also the methyl protons of 1M7AI are coupled with platinum ($^{5}J_{H,Pt} = 10$ Hz) (Figure S2). A similar through space coupling has been observed in few other cases.[18-20] That this is a clear case of through-space coupling is also witnessed by the absence of detectable coupling between Pt and H_5 which are separated by four bonds, one less than the 1-Me protons.

X-ray Structure. An ORTEP drawing of the X-ray structure of complex **1** is shown in Figure S3, while the association of two molecular units in a dimeric aggregate is shown in Figure 2. Selected bond lengths and angles are given in Table 1.

The Pt atom has the expected square planar coordination geometry, and the donor atoms are two chlorides and two nitrogens in cis positions. The 1M7AI ligand is planar, and his plane is nearly perpendicular to that of coordination [dihedral angle of 82.2(1)°] so to minimize steric interactions with cis ligands.

The platinum-ligand distances (2.045[7] Å for Pt−N and 2.300[2] Å for Pt−Cl) are in good agreement with values found in structurally related complexes.[21, 22] The C−N and C−C distances in the heteroaromatic ligand are in accord with an extensive π-bond delocalization.[23] The complex displays a very short platinum-methyl distance [Pt···C(10) distance of 3.203(8) Å]; moreover one methyl proton is directed toward the platinum atom indicating a H-bond type interaction [Pt···H(C) distance of 2.78(9) Å with a Pt···H–C angle of 105°], as confirmed by the downfield shift of the methyl protons with respect to the uncoordinated ligand. In previous reports, similar M···H–C interactions for d^8 square–planar complexes have been described as anagostic interactions.[24] The same methyl forms a hydrogen bond with the chloride of another complex unit related by an inversion center (Figure 2). Within the dimeric aggregate, two methyl groups are placed at very short distance [C8···C8(-*x* + 1, -*y*, -*z*+1) distance of 3.11(1) Å)], much shorter than the sum of the van der Waals radii (3.40 Å).[25] Other C···C distances shorter than the sum of van der Waals radii have been found for methyl groups in the IsoStar database.[26] However in those cases (three) the C···C distances were longer (between 3.2 and 3.3 Å) than in the present case.[27-29]

The H atoms of the methyl group (found in the difference Fourier map) exhibit N−C−H angles smaller than 109° (N1−C10−H10A, N1−C10−H10B, and N1−C10−H10C angles of 104°, 108°, and 104°, respectively), arguably connected to H-bond formation (HA and HC) and short C10···C10 distance. Intermolecular interactions between amminic groups and chlorido ligands of different molecular units (the chlorido not involved in interaction with the Me of 1M7AI) support the growth of the supramolecular assembly of complex molecules [N2···Cl1(x + ½, - y + ½, - z + 1) 3.388(7) Å, (N2)H22···Cl1(x + ½, - y + ½, - z + 1) 2.82(9) Å, N2−H22···Cl1(x + ½, - y + ½, - z + 1) 143(9)°; N2···Cl1(- x + ½+1, y - ½, z) 3.408(7) Å, (N2)H23···Cl1(-x + ½+1, y - ½, z) 2.69(9) Å, N2−H23···Cl1 (-x + ½ +1, y - ½, z) 139(9)°]. In Figure S4 a centrosymmetric tetrameric aggregate of four subunits is shown. In each tetrameric aggregate there are two non-classical C−H···π–C2−C3 contacts [distance between (C)H and π–C2−C3 centroid of 2.65 Å].

Cytotoxicity. The cytotoxic activity of **1** and **2** was determined against human ovarian carcinoma cell lines A2780 (cisplatin sensitive) and A2780cisR (with acquired resistance to cisplatin) and human breast adenocarcinoma cells MCF-7 (inherently resistant to cisplatin) commonly used to test cytotoxic activity of cisplatin derivatives and other antitumor metallodrugs. The tumor cell lines were incubated with the platinum compounds and cell survival in cultures was evaluated as described in the "Experimental section". The IC_{50} values (concentration of compound which causes death in 50 % of cells) for 72 h of treatment are reported in Table 2; both compounds showed promising activity.

The activity of **1** and **2** was comparable to that of cisplatin in cisplatin-sensitive A2780 cells, but, importantly, both compounds were markedly more toxic than cisplatin in cisplatin-resistant A2780cisR cells and also moderately more toxic in MCF-7 (inherently resistant to cisplatin). Furthermore, the resistance factor [defined as the ratio of IC_{50} values in resistant (A2780cisR)

and cisplatin-sensitive parent cells (A2780)] was 1.0 and 0.8 for **1** and **2**, respectively, whereas it was markedly higher for cisplatin (6.5). This suggests that the mechanism underlying the biological action of **1** and **2** is somewhat different from that of cisplatin allowing both compounds to overcome successfully the resistance mechanisms operating in the case of cisplatin.

Apart from the ability of compounds **1** and **2** to kill tumor cells resistant to clinically used cisplatin, the low cytotoxicity of **1** in noncancerous human primary skin fibroblasts demands special attention. The IC_{50} of **1** in noncancerous cells is more than one order of magnitude greater than that found for the sensitive cancer cell line A2780. Thus, **1** shows selectivity for tumor cells relative to the nontumorigenic, normal, cells. Whereas the selectivity of **1,** in comparison with that of cisplatin, is less in A2780 cells (sensitive to cisplatin), it is higher in A2780cisR cells (resistant to cisplatin). The trans derivative **2** is somewhat less selective than **1**. Nevertheless its design and synthesis represents a broadening of the database of Pt^{II}-dichlorido compounds in which the trans geometry can be effectively activated by the replacement of one NH_3 ligand in transplatin by a planar N-donor heterocycle.[5, 6]

Cellular Accumulation. One of the key factors affecting the biological activity of metal-based drugs is the intracellular accumulation. To examine the ability of **1** and **2** to enter and accumulate in tumor cells, A2780, A2780cisR and MCF-7 cells were incubated with the platinum complexes for 5 and 24 h exposure times (A2780 cells were incubated with the platinum complexes also for 3 and 12 h) and the platinum content measured. The amount of platinum associated with the cells after only 10 s of treatment was also determined and assumed to represent the level of platinum adsorption on the cells surface. These values were subtracted from those found after 3, 5, 12 or 24 h exposure time. The platinum adsorption on the cell surface was approximately the same for **1** and **2** and very similar to that of cisplatin (not shown).

The intracellular accumulation of platinum from **1** was somewhat lower than that from cisplatin in cisplatin sensitive A2780 cells while it was very similar to that from cisplatin in resistant A2780cisR cells. Therefore the enhanced cytotoxic activity of **1** toward A2780cisR cells is not due to enhanced accumulation of the drug. Unlike A2780 and A2780cisR cells, the accumulation of platinum from **1** was greater than that from cisplatin in MCF-7 cells (2-3-fold increase), in agreement with the greater cytotoxicity of **1** toward this cell line.

Unlike **1**, the cellular uptake of platinum from **2** was markedly greater (3 - 9-fold) than that from cisplatin in all cell lines tested in this work (Table 3, S1 and Figure S5). This is in line with the lower polarity of trans compounds as compared to cis ones. A scale of hydrophobicity can be deduced from log P values for octanol/water partition. Log P values for cisplatin, **1** and **2** were -1.92,[30] -0.66 and 0.83, respectively, confirming that **2** is the most hydrophobic.

Notwithstanding the greater accumulation, the toxicity of **2** in these cancer cells is comparable to that of **1** (Table 2). For compounds **1** and **2**, the cellular uptake of platinum from these complexes remains constant for sensitive and resistant ovarian cancer cells as does their cytotoxicity. In contrast, the platinum uptake from cisplatin decreases by 1.5 - 2-fold which results in an even greater decrease of cytotoxicity (6.5-fold). The greater toxicity of **1** and **2** toward MCF-7 cells, which are intrinsically resistant to cisplatin, correlates with a greater degree of platinum uptake from these drugs.

DNA Platination in Cells Exposed to Platinum Complexes. The cytostatic action of conventional platinum anticancer drugs is connected with their binding to nuclear DNA. The degree of DNA platination was determined by inductively coupled plasma mass spectrometry (ICP-MS) for the three cell lines after their exposure to **1**, **2** or cisplatin for 5 or 24 h (Table 4). The amount of platinum associated with DNA, expressed as percentage of total platinum accumulated in the cell, are reported in Table 5.

After both 5 h and 24 h exposure times, the degree of DNA platination was comparable for **1** and cisplatin. Notably, while for cisplatin the degree of DNA platination was considerably lower in cisplatin-resistant A2780cisR cells than in sensitive A2780 cells, this was not the case for complex **1**. These accumulation studies also suggest that in the case of cisplatin the reduced toxicity toward A2780cisR cells is only in part due to reduced drug uptake, while there will be also a contribution from a higher rate of DNA repair, as clearly shown by the results at 24 h incubation time. Overall, DNA platination appears to correlate with toxicity of **1** and cisplatin in the tumor cell lines tested in this work. This finding is in good agreement with the view that, similarly to cisplatin, DNA is the biological target also for **1**.

Notably, for all cell lines and for all exposure times the amount of platinum bound to DNA was significantly greater (1.5 - 9-fold) in the case of **2** than in the case of **1**. However, **2** had a cytotoxicity comparable to that of **1** (Table 3). Assuming that DNA is the pharmacological target also for Pt complexes with 1M7AI, these results suggest that **2** modifies DNA differently from **1** and that DNA adducts of **2** are inherently less effective than those of **1.**

From the data reported in Tables 3 and 4 it is possible to estimate the percentage of total platinum in the cells that binds to DNA. In most cases the percentage is lower for **2** than for **1** or cisplatin (Table 5).

DNA and Protein Binding in Cell-free Media. The experiments described above reveal that for **1** and **2** there is no direct correlation between the intracellular accumulation and the degree of DNA platination. To clarify this result, the DNA binding properties of **1** and **2** were studied in cell free media and compared to that of cisplatin. The platinum complexes were incubated with calf thymus (CT) DNA at r_i (r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA) of 0.05 in $NaClO_4$ (10 mM) at 310 K in the dark. Aliquots were withdrawn at various time intervals, the free (unbound) platinum

compound was removed by exhaustive dialysis, and the content of platinum and the concentration of DNA were determined by flameless atomic absorption spectrometry (FAAS) and absorption spectrophotometry, respectively. The results, shown in Figure 3A, indicate that after 24 h of incubation the platinum bound to DNA is 69 ± 4% and 95 ± 3 % for **1** and **2**, respectively (cisplatin binds to DNA quantitatively). These DNA binding experiments indicate that irreversible binding to DNA is somewhat slower for **1** than for **2** and cisplatin. Therefore, the lower portion of platinum bound to nuclear DNA, with respect to the total platinum taken up by the cell after 24 h, in the case of **2** cannot be a consequence of its lower DNA binding affinity. In other words, other factors, such as greater trapping of more lipophilic **2** into the cell membrane or greater reactivity of the trans compound toward intracellular nucleophiles other than DNA (such as sulfur-containing species) may play a role.

The possibility that the intracellular pool of **1** and **2** that can reach and bind DNA can be reduced differently for these platinum complexes by interaction with intracellular nucleophiles was tested by investigating the interaction of **1** and **2** with HSA. Therefore, **1** or **2** were incubated with HSA at molar ratio 1:1 in Tris.HCl (10 mM, pH 7.4) and in the presence of NaCl (8 mM) at 310 K in the dark. Aliquots were withdrawn at various time intervals, the free (unbound) platinum compound was removed by exhaustive dialysis, and the content of platinum and the concentration of HSA were determined by FAAS and absorption spectrophotometry, respectively. The reaction of **2** with HSA was noticeably faster than that of **1 (**Figure 3B). Based on this outcome, we can hypothesize that a significantly higher fraction of **2** is trapped in the cytoplasm by reaction with intracellular proteins, peptides and/or other intracellular thiols and cannot access DNA. The higher reactivity of **2** towards the biomolecules present in the cytoplasm can thus be responsible for its lower efficiency to platinated DNA despite its higher accumulation in the cell.

Transcription Mapping. Samples of DNA (the NdeI/HpaI fragment of pSP73KB plasmid) modified by **1** or **2** for analysis by transcription mapping were prepared in $NaClO_4$ (10 mM) at 310 K. After 24 h incubation with the complex, the DNA sample was precipitated with ethanol, then redissolved in the medium necessary for the transcription mapping analysis, while the r_b value (the number of molecules of platinum complex bound per nucleotide residue) in these samples was determined by platinum FAAS and by measurement of absorbance at 260 nm. By adopting this procedure, the transcription mapping analysis (described below) is performed in the absence of unbound (free) Pt complex.

Cutting of pSP73KB DNA by NdeI and HpaI restriction endonucleases yields a 212-base pairs (bp) fragment (a substantial part of its nucleotide sequence is shown in Figure 4B). This fragment contains a T7 RNA polymerase promotor. This DNA template was modified by **1** or **2** at $r_b = 0.01$ and its ability to terminate the *in vitro* RNA synthesis by T7 RNA polymerase at the level or in the proximity of platinum-DNA adducts was evaluated (Figure 4A). The major stop sites observed for **1** correspond to the sites for cisplatin, indicating similar DNA binding modes for both cis compounds. On the other hand, the pattern of stop sites obtained for compound **2** is similar to that found for transplatin (e.g. much less regular with respect to those of the cis compounds, Figure 4B).

Localization in Tumor Cells. A2780 cells were first exposed to **1** or **2** (30 μM) for 5 h and then visualized by confocal microscopy (Figure 5). After the treatment, cells were carefully washed with platinum-free medium so that no residual free platinum complex was present in the growing medium. No signal was yielded by the untreated cells (Figure 5C) or the cells treated with free 1M7AI (Figure 5F) or cisplatin (not shown), which suggests that the signal yielded by the cells treated with **1** or **2** comes mainly from the platinum complex containing 1M7AI ligand. The resulting images show that the subcellular localization of the light is largely non-nuclear.

This is in good correlation with previously described data showing that only about 1% of total cell-associated platinum is localized on nuclear DNA. The signal appears to be localized mostly in cell organelles (for example in lysosomes or late endosomes) rather than on cytoplasmic membranes (Figures 5A,B). Data shown in Figures 5D,E are consistent with the thesis that the intracellular accumulation of **1** and **2** increases with the time of treatment and that the intracellular accumulation of **2** is significantly higher than that of **1**.

Cell Cycle Analysis. To further characterize the cytotoxic effect of **1** and **2**, an analysis of the cell cycle perturbations was performed in A2780 cells exposed to **1**, **2** or to the reference compound cisplatin. Activation of cell cycle checkpoints is a general cellular response to cytotoxic agents. Previous studies have indicated that cisplatin and other platinum agents predominantly inhibit cell cycle progression at the S and/or G_2 phase.[31, 32] Cells were exposed to **1**, **2**, or cisplatin at their IC_{50} concentrations (Table 2) for 24 or 72 h. Significant differences in cell cycle modulation between **1** and **2** were observed already after 24 h of treatment and the differences became more pronounced as the treatment time increased (Figure 6).

Exposure of A2780 cells to **1**, **2**, or cisplatin led to the appearance of a population in the sub-G_1 region (more evident after 72 h exposure), where apoptotic and necrotic cells are found. Therefore, the appearance of a sub-G_1 peak is consistent with the onset of internucleosomal DNA cleavage in late apoptosis.[32] Importantly, treatment with **2** affected the G_0/G_1 populations less than treatment with **1** or cisplatin. Unlike **2**, **1** and cisplatin caused significant accumulation of cells in the S-phase of the cell cycle at short exposure time (24 h) and cell cycle block in G_2-phase at long exposure time (72 h). These results are in agreement with data previously reported for cisplatin[32, 33] and highlight the similarity between cell cycle perturbations induced by **1** and cisplatin whereas **2**, having trans geometry, behaves differently.

Detection of Apoptosis and Necrosis. The levels of apoptosis and necrosis induced in A2780 cells by **1**, **2** or cisplatin at various concentrations over 24 and 72 h exposure time was quantified by the cell death detection ELISA colorimetric kit. The assay is based on the quantitative "sandwich enzyme immunoassay" principle using mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes (histone-associated DNA fragments) in the cytoplasmic fraction of the cell lysates. The relative amounts of cytoplasmic histone-associated DNA fragments are measured after the induction of apoptosis or when these fragments are released from necrotic cells. Figure 7 shows DNA fragmentation induced by **1**, **2**, or cisplatin in A2780 cells.

The results demonstrate that treatment with all tested antitumor agents leads to concentration dependent apoptotic events in the A2780 cell line (Figure 7A). Importantly, **1** induces a lower level of DNA fragmentation, due to apoptosis, in comparison with cisplatin over the whole range of concentrations used. In contrast, **2** was less effective in inducing DNA fragmentation at low concentration (IC_{20}) compared to cisplatin, whereas at higher concentrations (corresponding to IC_{50} and IC_{80}) it was more effective than cisplatin. A similar ELISA assay was also used to detect the extent of necrosis induced by **1**, **2**, or cisplatin. Importantly, the level of necrosis was significantly lower compared to that of apoptosis triggered by **1**, **2**, or cisplatin (Figure 7B). At long treatment times, apoptosis predominates over necrosis, with **2** being less efficient in inducing apoptosis compared to the cis-compounds **1** and cisplatin (Figure 7B). The data are consistent with the results of flow cytometric measurements (Figure 6) where **2** was the most effective in inducing sub G_1 population of cells after 24 h of incubation, but the least efficient after 72 h.

To further explore this finding, we determined the cytotoxicities of **1**, **2** and cisplatin also after a short time of treatment. After 24 h of incubation of A2780 cells with the Pt(II) complexes, the

IC_{50} values assessed by the MTT test [MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were 47 ± 2, 9.4 ± 0.8, and 14.9 ± 0.9 μM for **1**, **2**, and cisplatin, respectively. These data also confirm that **2** is more effective in the early phases of the treatment, whereas the cytotoxic effect of **1** is manifested mostly after longer period of incubation (Figure 8).

The Involvement of p53 Pathway in the Mechanism of Action. The promotion of apoptosis by the platinum complexes may be connected with the induction of tumor suppressor p53, which plays a pro-apoptotic role.[Amaral, 2010 #6433] To investigate the possible involvement of p53 pathway in the mechanism of action of **1** and **2**, the effect of these platinum complexes on the viability of human colon carcinoma cells expressing p53 (HCT116 $p53^{+/+}$) or non expressing p53 (HCT116 $p53^{-/-}$) was investigated. The cells were treated with **1**, **2** or (for comparative purposes) cisplatin for 72 h as described in the Experimental section. The IC_{50} values and DNA content in the treated cells were determined as described in a previously published article.[34] The data showed that cisplatin and **1** significantly reduced the number of cells in a dose-dependent manner, being more effective in p53-proficient cells compared to the p53-knock-out cells (Table 2 and Figures 9A,B). These results suggest that transcription factor p53 plays an important role in the mechanism of action of **1**. In this regard, the mechanism of action of **1** appears to be similar to that of cisplatin, which was shown to be more effective in p53-wild type over the p53-mutant tumor cells.[35] In contrast, the effectivity of **2** in killing $p53^{+/+}$ and $p53^{-/-}$ cells was not too different (Table 2 and Figure 9C) indicating that **2** operates via a pathway that is p53-independent. That our new transplatin derivative **2** operates via a p53-independent pathway is in a fundamental contrast to what has been reported for other antitumor-active derivatives of transplatin, namely transplatinum complexes containing a planar, N-donor, heterocycle.[36] Thus, this is to the best of our knowledge the first demonstration that p53 does not play a role in the toxic effects induced in

tumor cells by the trans-platinum drugs. A plausible implication of this finding is that **2** might be active *in vivo* in contrast to other activated transplatinum complexes hitherto tested *in vivo*. The work is in progress to test this challenging hypothesis.

CONCLUSIONS

Two new bifunctional and mononuclear Pt(II) compounds, having cis- and trans- geometry and containing the 1-methyl-7-azaindole ligand, were synthesized, fully characterized, screened for their toxicity against cancer cell lines, evaluated for their efficiency to enter the cells, damage DNA, affect cell cycle, and induce apoptosis. When compared with cisplatin, substitution of 1-methyl-7-azaindole for ammine results in an increase of the toxic efficiency and selectivity for tumor cells in cisplatin-resistant cancer cells. On the other hand, such a substitution activates the trans geometry so that the cytotoxicity of the trans isomer, compared to that of cisplatin, is nearly equivalent in cisplatin sensitive tumor cells and markedly better in cisplatin resistant tumor cells. The ability to overcome resistance to cisplatin appears to be associated with an increased level of cellular DNA platination by these compounds as compared to cisplatin. Although the trans isomer enters cells more easily due to its higher lipophilicity, it is also more easily trapped in the cytoplasm by faster reactions with intracellular nucleophiles when compared with its cis congener, with consequent reduction of binding to cellular DNA. For both isomers the cytotoxic effect is accompanied by triggering of apoptosis and cell cycle arrest in S and G_2/M phases. Interestingly, whilst the trans isomer demonstrates its biological action in the early phases of the treatment, the cytotoxic effect of the cis isomer is more pronounced after longer incubation times.

The differences in the biological (cytotoxic) activities of **1** and **2** may be associated with their different DNA binding mode, different capability to induce cell cycle perturbations and fundamentally different role of transcription factor p53 in their mechanism of action. Notably, both isomers make it possible to detect their cellular uptake and distribution in living cells by confocal microscopy without their modification with an optically active tag. Moreover, our current work not only reinforces the potential utility of the trans-platinum compound to act as antitumor drugs, but also indicates that transplatin derivatives containing a 7-azaindole ligand can be subjected to different chemical modifications resulting in different biological properties, therefore representing an ideal starting point for further developments.

EXPERIMENTAL SECTION

Commercial reagent grade chemicals and solvents were used as received without further purification. Cisplatin, DMF, HSA, and propidium iodide were from Sigma-Aldrich (Prague, Czech Republic). 1-Methyl-7-azaindole was from Ark Pharm, Inc. (Libertyville, USA). CT DNA (42% G+C, mean molecular mass ca. $2x10^7$) was prepared and characterized as described previously.[37, 38] Plasmid pSP73KB (2455 bp) was isolated according to standard procedures. Restriction endonucleases were purchased from New England Biolabs. Acrylamide and bis(acrylamide) were obtained from Merck KgaA (Darmstadt, Germany). MTT was from Calbiochem (Darmstadt, Germany). [α-^{32}P]CTP was obtained from MP Biomedicals, LLC (Irvine, CA). RNase A was from Qiagen (USA). DNazol (genomic DNA isolation reagent) was obtained from MRC (Cincinnati, OH). RPMI 1640 medium, fetal bovine serum (FBS), trypsin/EDTA, and Dulbecco's modified Eagle's medium (DMEM) were from PAA (Pasching, Austria). Penicilin, gentamycin and streptomycin were from Serva (Heidelberg, Germany).

Riboprobe Gemini System II for transcription mapping containing T7 RNA polymerase was purchased from Promega (Madison, WI). Cell death detection ELISA plus kit was from Roche Molecular Biochemicals (Mannheim, Germany).

Synthesis. $K[PtCl_3(NH_3)]$ and cis-$[PtI_2(NH_3)_2]$ were prepared according to already reported procedures.[15, 39] The elemental analysis and the spectroscopic features were fully consistent with the data reported in the literature.

*Cis-[$PtCl_2(NH_3)$(1M7AI)] (**1**).* $K[PtCl_3(NH_3)]$ (0.26 g, 0.71 mmol) was suspended in CH_3OH (40 mL) and treated with a slight excess of 1M7AI (0.115 g, 0.84 mmol). The mixture was kept under stirring at room temperature for 4 days and then the suspension was filtered through Celite and the pale yellow solution was evaporated to dryness under reduced pressure. The solid residue was triturated with CH_2Cl_2 (8 mL) in order to remove the excess of ligand. The obtained suspension was filtered off and the resulting yellow solid was dried under vacuum, washed with warm water (328 K) to remove KCl, dried again under vacuum and analyzed by TLC using dichloromethane/acetone 1:1 as eluent. The chromatogram showed one single spot, corresponding to **1**, in addition to some non-eluting side products. Therefore, the raw material was subjected to silica gel chromatography, using dichloromethane/acetone 1:1 as eluent. Were obtained 0.14 g (0.34 mmol, 48% yield) of **1**. Anal. Calculated for *cis*-$[PtCl_2(NH_3)$**(**1M7AI)$]\cdot CH_3OH$ ($C_8H_{11}N_3Cl_2Pt\cdot CH_3OH$): C, 24.16; H, 3.38; N, 9.40%. Found: C, 23.83; H, 2.99; N, 9.65%. ^{1}H-NMR (acetone-d_6, numbering scheme of 1M7AI as in Schemes S1 and S2) δ: 8.89 (m, 1 H, H_6), 8.06 (m, 1 H, H_4), 7.55 (m, 1 H, H_2), 7.10 (m, 1 H, H_5), 6.63 (d, 1 H, H_3), 5.24 (s, 3 H, CH_3), 4.10 (b, 3 H, NH_3) ppm. ^{195}Pt-NMR (acetone-d_6) δ: −1,970 ppm. ESI-MS: calculated for $[C_8H_{11}N_3Cl_2PtNa]^+$ 437.98. *Found: m/z* (% relative to the base peak) 437.9 (100), $[M + Na]^+$.

*Trans-[PtCl$_2$(NH$_3$)(1M7AI)] (**2**).* Compound **2** was prepared by a two-step process which involved the preparation of the intermediate species *trans*-[PtI$_2$(NH$_3$)(1M7AI)]. *Trans*-[PtI$_2$(NH$_3$)(1M7AI)] was prepared from *cis*-[PtI$_2$(NH$_3$)$_2$] and 1M7AI. *cis*-[PtI$_2$(NH$_3$)$_2$] (1.01 g, 2.09 mmol) was reacted with AgNO$_3$ (0.71 g, 4.19 mmol) in H$_2$O (50 mL) and kept at 328 K in the dark for 20 min. The AgI precipitate was separated by filtration and the clear solution treated with a twofold excess of 1M7AI (0.55 g, 4.19 mmol). The mixture was heated at 343 K for 3 h; then the suspension was filtered through Celite and the yellow mother solution containing *cis*-[Pt(NH$_3$)$_2$(1M7AI)$_2$]$^{2+}$ was treated with an aqueous solution of KI (1.74 g, 1.05 mmol), added dropwise over a period of 5 min. The solution was stirred at 343 K for 2 h, meanwhile a brown precipitate formed. The brown solid was collected by filtration of the mother liquor and left to dry under vacuum. The solid residue was triturated with a mixture of acetone/dichloromethane (1:1, v/v; 100 mL), the solution was separated by filtration, the solvent was evaporated under reduced pressure, and the solid residue analyzed by TLC using dichloromethane/acetone 9:1 as eluent. The chromatogram showed two spots, corresponding (in order of increasing R$_f$) to *trans*-[PtI$_2$(NH$_3$)(1M7AI)] and *trans*-[PtI$_2$(1M7AI)$_2$]. Therefore, the raw material was subjected to silica gel chromatography, using dichloromethane/acetone 9:1 as eluent. Were obtained 0.250 g (0.42 mmol, 20% yield) of *trans*-[PtI$_2$(NH$_3$)(1M7AI)]. Anal. Calculated for *trans*-[PtI$_2$(NH$_3$)(1M7AI)] (C$_8$H$_{11}$N$_3$I$_2$Pt): C, 16.05; H, 1.84; N, 7.02%. Found: C, 16.65; H, 1.91; N, 6.89%. ^{1}H-NMR (acetone-d_6, numbering scheme of 1M7AI as in Schemes S1 and S2) δ: 8.72 (m, 1 H, H$_6$), 7.97 (m, 1 H, H$_4$), 7.50 (m, 1 H, H$_2$), 7.03 (m, 1 H, H$_5$), 6.59 (d, 1 H, H$_3$), 5.17 (s, 3 H, CH$_3$), 3.88 (b, 3 H, NH$_3$) ppm. ^{195}Pt-NMR (acetone-d_6) δ: −3,240 ppm. *ESI-MS:* calculated for [C$_8$H$_{11}$N$_3$I$_2$PtNa]$^+$ 621.07. *Found: m/z* (% relative to the base peak) 621.05(100), [M + Na]$^+$.

The dichlorido complex **2** was prepared from *trans*-[$PtI_2(NH_3)$(1M7AI)] (0.11 g, 0.18 mmol) dissolved in H_2O (50 mL) and treated with $AgNO_3$ (0.060 g, 0.37 mmol). After 3 h stirring at 323 K and in the dark, the solution was filtered (to remove the AgI precipitate), treated with an excess of KCl (0.13 g, 1.8 mmol) and kept at 328 K for 1 h, during which time a brown solid precipitated from the solution. The suspension was concentrated to a minimum volume under reduced pressure, while keeping the reaction vessel at 328 K, and then cooled to 277 K in order to promote further precipitation of the desired product. The solid residue was transferred on a glass filter, washed with a minimum amount of cold water, dried under vacuum and analyzed by TLC using dichloromethane/acetone 9:1 as eluent. The chromatogram showed one single spot, corresponding to **2**, in addition to some non-eluting side products. Therefore, the raw material was subjected to silica gel chromatography, using dichloromethane/acetone 9:1 as eluent. Were obtained 0.037 g (0.089 mmol, 50% yield) of **2**. Anal. Calculated for *trans*-[$PtCl_2(NH_3)$(1M7AI)]·¼CH_3COCH_3 ($C_8H_{11}N_3Cl_2Pt$·¼CH_3COCH_3): C, 24.44; H, 2.91; N, 9.78%. Found*:* C, 24.70; H, 2.92; N, 10.09%. ^{1}H-NMR (acetone-d_6, numbering scheme of 1M7AI as in Schemes S1 and S2) δ: 8.71 (m, 1 H, H_6), 8.03 (m, 1 H, H_4), 7.51 (m, 1 H, H_2), 7.09 (m, 1 H, H_5), 6.61 (d, 1 H, H_3), 5.17 (s, 3 H, CH_3), 3.85 (b, 3 H, NH_3) ppm. ^{195}Pt-NMR (acetone-d_6) δ: −1,967 ppm. *ESI-MS:* calculated for [$C_8H_{11}N_3Cl_2PtNa$] 438.18. *Found: m/z* (% relative to the base peak) 437.9(100), $[M + Na]^+$.

X-ray crystallography. Crystals suitable for X-ray investigation were obtained from water/acetone (1:2 ratio). Reflections were collected with Mo-*K*α radiation by using a Bruker AXS X8 APEX CCD System. All reflections were indexed, integrated, and corrected for Lorentz, polarization, and absorption effects using the program SADABS [Sheldrick G. M., SADABS University of Göttingen Germany (1996)]. Data collection, data reduction, and unit cell

refinement were carried out with the SAINT-IRIX package [Bruker: SAINT-IRIX BrukeRAXSInc Madison Wisconsin USA (2003)].

The model was refined by full-matrix least-square methods. All non-hydrogen atoms were refined anisotropically. The H atoms of the amino and methyl groups were found in a difference Fourier map and were refined with Uiso(H) = 1.5 Ueq(parent). Hydrogen atoms of aromatic rings were placed at calculated positions and refined given isotropic parameters equivalent to 1.2 times those of the atom to which they are attached. All calculations and molecular graphics were carried out using SIR2004,[40] SHELXL97,[41] PARST97,[42, 43] WinGX,[44] and ORTEP-3 for Windows packages.[45] Details of the crystal data are listed in Table 6.

Crystallographic data (without structure factors) have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 1024413. Copies of the data can be obtained free of charge from the CCDC (12 Union Road, Cambridge CB2 1EZ, UK; tel: (+44) 1223-336-408; fax: (+44) 1223-336-003; e-mail: deposit@ccdc.cam.ac.uk; website http://www.ccdc.cam.ac.uk/data_request/).

Cell Lines and Culture. The human ovarian carcinomas A2780 (parent cisplatin-sensitive) and A2780cisR (cisplatin resistant variant of A2780) and the human breast adenocarcinoma MCF-7 (cisplatin in-born resistance) cell lines were supplied by Professor B. Keppler, University of Vienna (Austria). The human colon carcinoma HCT116 (parental, $p53^{+/+}$) and its p53-null derivative (p53-/-) cells[46] were a kind gift of Dr. M. Brazdova, Institute of Biophysics, Brno (Czech Republic). The A2780 and A2780cisR were grown in RPMI 1640 medium supplemented with streptomycin (100 μg mL^{-1}), penicilin (100 U mL^{-1}) and heat inactivated FBS (10%). The acquired resistance of A2780cisR cells was maintained by supplementing the medium with cisplatin (1 μM) every second subculture. The MCF-7, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were grown in DMEM supplemented with streptomycin (100 μg mL^{-1}), penicilin (100 U mL^{-1})

and heat inactivated FBS (10%). The cells were cultured in a humidified incubator at 310 K in an atmosphere of 5% CO_2 and subcultured 2−3 times per week with an appropriate plating density. Human skin fibroblasts (primary cell culture) was a kind gift from Professor T. Adam, Laboratory of Inherited Metabolic Disorders, Department of Clinical Chemistry, Palacky University and Hospital, Olomouc, Czech Republic. These cells were grown in DMEM supplemented with streptomycin (100 μg mL^{-1}), penicilin (100 U mL^{-1}) and 10% heat inactivated FBS.

***In Vitro* Growth Inhibition Assay.** Influence of platinum complexes on cell viability was tested using the colorimetric MTT assay. Cells were seeded on 96-well tissue culture plates at a density of 10^4 cells/well in 100 μL of growth medium and incubated at 310 K in a humidified 5% CO_2 atmosphere for 16 h (overnight). After the incubation period the cells were treated with the compounds under investigations and kept in the incubator for additional 72 h. The stock solutions of platinum compounds were always freshly prepared in DMF before use. The final concentration of DMF in cell culture medium did not exceed 0.1% (v/v), which was shown not to affect cell growth. The final concentrations of tested compounds were in the range of 0 to 80 μM in a volume of 200 μL/well. Subsequently, 10 μL of MTT solution (5 mg mL^{-1}) was added to each well, and plates were incubated for 4 h. At the end of the incubation time the medium was removed and the formazan product was dissolved in 100 μL of DMSO per well. Cell viability was evaluated by measuring the absorbance at 570 nm (reference wavelength at 630 nm) using an Absorbance Reader Sunrise Tecan Schoeller. IC_{50} values were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were done in triplicate. The reading values were converted to the percentage of control (% cell survival). Cytotoxic effects were expressed as IC_{50}.

Cellular Accumulation. Cellular uptake of **1**, **2**, and cisplatin was measured in A2780, A2780cisR and MCF-7 cell lines. The cells were seeded on 100 mm tissue culture dishes ($3x10^6$ cells/dish in 10 mL of growth medium). After 48 h of incubation, the cells were treated with tested compounds (at their IC_{50} values) for 10 s, 3, 5, 12 or 24 h (the concentrations were verified by measuring the platinum content in the growing medium by FAAS). The attached cells were harvested by trypsinization and the cell pellets washed twice with cold PBS (277 K). The pellets were digested by using microwave acid (HCl, 11 M) digestion system (MARS5, CEM) to give a fully homogenized solution. Final platinum content in the samples was determined by ICP-MS. The results of cellular platinum uptake were corrected for adsorption effects.[47]

Determination of Partition Coefficients of Platinum Complexes. Partition coefficients of **1** and **2** were determined in an octanol/saline system. Briefly, saline solution (100 mM NaCl) and n-octanol were pre-saturated with n-octanol and saline, respectively; then **1** or **2** (10^{-3} g, 2.41 x 10^{-3} mmol) was dissolved in saline (3 mL and 10 mL for **1** and **2**, respectively) at 310 K and the complex concentration was determined by FAAS. Equal volumes of the drug solution and n-octanol were placed in a glass tube and the mixture shaken mechanically for 1 h at 310 K. Then the mixture was centrifuged (1500 rpm, 5 min) and the bottom aqueous layer was removed and subjected to platinum analysis by FAAS. The complex concentration in the n-octanol phase was calculated by the difference between the complex concentration in saline before and after n-octanol extraction. Each value reported in the text represents a mean of triplicate determination; differences between the mean and individual values were <5%.

DNA Platination in Cells Exposed to Platinum Complexes. A2780, A2780cisR or MCF-7 cells grown to near confluence were treated with **1**, **2** or cisplatin at IC_{50} values and incubated for 5 or 24 h. After incubation, the cells were trypsinized and washed twice in ice-cold PBS. Cells were lysed in DNAzol (DNAzol genomic DNA isolation reagent) supplemented with RNase A

(100 μg mL^{-1}). The genomic DNA was precipitated from the lysate with ethanol, dried, and resuspended in water. The DNA content in each sample was determined by UV spectrophotometry. To avoid the effect of high DNA concentration on detection of platinum in the samples, the DNA samples were digested in the presence of HCl (11 M) using a high pressure microwave mineralization system. Experiments were performed in triplicate, and the values are the means ± SD.

Platination Reactions in Cell-free Media. CT or plasmid DNAs were incubated with the platinum complexes in $NaClO_4$ (10 mM) at 310 K in the dark. After 24 h, the samples were exhaustively dialyzed against the medium required for subsequent biochemical or biophysical analysis. An aliquot of these samples was used to determine r_b values by FAAS. Similarly, HSA was incubated with the platinum complexes (molar ratio of 1:1) in Tris.HCl (10 mM, pH 7.4) plus NaCl (8 mM) at 310 K in the dark. Aliquots were withdrawn at various time intervals, quickly cooled to 193 K and exhaustively dialyzed against water. The platinum content in these samples was determined by FAAS, while the concentration of HSA was determined by absorption spectrophotometry.

DNA Transcription by RNA Polymerase *In Vitro.* Transcription of the pSP73KB DNA with T7 RNA polymerase and electrophoretic analysis of the transcripts were performed as previously described.[48] The concentration of DNA used in this assay was $3.9x10^{-5}$ M (relative to the monomeric nucleotide content).

Cell Cycle Analysis. A2780 cells were seeded on 60 mm tissue culture dishes at a density of $2x10^6$ cells/dish in 6 mL of growth medium and incubated at 310 K in a humidified 5% CO_2 atmosphere overnight. After incubation, the cells were treated with the compounds at IC_{50} values and stored at culture conditions. After 24 h, floating cells were collected, and attached cells were harvested by trypsinization (trypsin/EDTA in PBS). Total cells (floating + attached) were washed

twice in PBS (277 K), fixed in 70% ethanol, and stored at 253 K. Cell pellets were subsequently rinsed with PBS buffer and the sediment was stained with solution of propidium iodide (50 μg mL^{-1}) supplemented with RNase A (10 μg mL^{-1}) for 30 min at room temperature in the dark. The DNA content of the cells was analyzed using flow cytometry (Cell lab quanta TM SC-MPL, Beckman Coulter). The percentages of cells in individual cell cycle phases were analyzed using Multicycle AV for Windows (Phoenix Flow Systems, USA).

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.[49] In this assay, internucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes. The cells were seeded on 96-well tissue culture plates at a density of 10^4 cells/well. The treated A2780 cells were centrifuged (200 g, 10 min, room temperature), 20 μL of the supernatant (necrotic fraction) was used in the ELISA for detection of necrosis. A2780 cells were resuspended in 200 μL of the lysis buffer supplied by the manufacturer and incubated for 30 min at room temperature. After pelleting of the nuclei (200 g, 10 min, room temperature), 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 nm (reference wavelength was 490 nm) with a microplate reader (absorbance reader Tecan INFINITE M200, 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.

Confocal Microscopy. A2780 cells were seeded on glass bottom dishes (P50G-0-30-F, MatTek Co., Ashland, USA) at the density $1.5x10^6$ cells per dish and incubated at 310 K in a

humidified 5% CO_2 atmosphere overnight. The cells were then treated with **1**, or **2**, or 1M7AI (30 μM concentration) for 1 or 5 h. After this period, the cells were washed twice with PBS and supplemented with fresh medium (without compound). The distribution of tested compounds in tumor cells was analyzed by confocal microscopy. Samples were visualized with confocal microscope Leica TSC SP-5 X, sequentially scanned with the objective HCX PL APO lambda blue 63.0x1.20 water UV, corrected with an appropriate beam path. Resolution of captured images was 512·512 pix scanned with 100 Hz frequency. Samples were illuminated with UV laser at 355 nm and the light returning from the samples was detected with highly sensitive hybrid detectors at wavelengths 710-760 nm (for other details, see Supporting Information, Section Determination of the Wavelength Used to Illuminate Cells Treated with the Platinum Complexes in Confocal Microscopy Experiments). Signal intensity associated with single cells was analyzed in the center of captured image (256·256pix) to omit potential objective aberrations. The experiment was repeated twice with three replicates of each sample. Settings of the imaging software (laser strength and gain) were kept constant within each experiment to allow for comparison of the light intensities of samples.

Involvement of p53 Pathway in the Mechanism of Action. The effect of p53 status on the cytotoxic activity of **1**, **2** and cisplatin was tested as already described[34] using two isogenic human colon cancer cells, wild type p53-containing HCT116 cells [parental cells ($p53^{+/+}$) and its p53-null derivative ($p53^{-/-}$)].[46] Briefly, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were seeded in 96well black plates (Corning) at the density 4000 cells/well in 100 μL of growth medium (DMEM) and incubated overnight (16 h). Then the cells were treated with **1**, **2** or cisplatin for 6 h, washed twice with PBS and cultured in drug-free medium for additional 66 h at 310 K in 5% CO_2 humidified atmosphere. DMF-supplemented DMEM was introduced to the untreated controls. After the incubation period, the medium was replaced with 0.1% triton X-100 in MQ

water. The cells were lysed under continuous shaking on ice for 30 min. Subsequently, the cells were freeze thawed for five 30 min cycles. An equal volume of 2x SYBR Green I was added to 100 µL of the lysate. DNA contents were measured with fluorescence reader Infinite 200 (TECAN) at excitation/emission wavelength 485/535 nm. Experiments were done in triplicate. The data were normalized to the DNA content in control, untreated cells.

Physical Measurements. NMR spectra were collected at 295 K on a Bruker AVANCE DPX 300 MHz nominal ^{1}H Larmor frequency. ^{1}H chemical shifts were referenced to TMS by using the residual protic peak of the solvent (acetone-d_6, or D_2O) as internal reference. One-dimensional ^{195}Pt spectra were acquired using ^{1}H decoupling sequences. ^{195}Pt chemical shifts were referenced to K_2PtCl_4 (1 M in water, $\delta = -1{,}614$ ppm). Elemental analyses were carried out on a CHN Eurovector EA 3011 equipment. ESI-MS analyses were performed on an Agilent 1100 series LC-MSD Trap system VL. Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out with a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. The analysis with the aid of ICP-MS was performed using Agilent 7500 (Agilent, Japan). The gels were dried and visualized using a FUJIFILM BAS 2500 bioimaging analyzer.

ASSOCIATED CONTENT

Supporting Information

"Supporting Information Available: Product characterization including NMR and fluorescence data, data on intracellular accumulation. This material is available free of charge via the Internet at http://pubs.acs.org."

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

1, *cis*-[$PtCl_2(NH_3)$(1M7AI)]; **2**, *trans*-[$PtCl_2(NH_3)$(1M7AI)]; 1M7AI, 1-methyl-7-azaindole; bp, base pair; cisplatin, *cis*-diamminedichloridoplatinum(II); CT, calf thymus; DMEM, Dulbecco's modified Eagle's medium; FAAS, flameless atomic absorption spectrometry; FBS, fetal bovine serum; IC_{50}, concentration of compound which causes death in 50 % of cells; ICP-MS, inductively coupled plasma mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide; picoplatin, *cis*-amminedichlorido(2-methylpyridine)platinum(II); r_b, the number of molecules of platinum complex bound per nucleotide residue; r_i, the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA; transplatin, *trans*-diamminedichloridoplatinum(II)

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Table 1. Selected Bond Lengths (Å) and Angles (°) for *cis*-[$PtCl_2(NH_3)$(1M7AI)] (1)

Pt1−N7	2.043(6)	N7− Pt1−N2	92.2(2)
Pt1−N2	2.047(4)	N2−Pt1−Cl2	88.5(2)
Pt1−Cl1	2.297(2)	N7−Pt1−Cl1	87.7(2)
Pt1−Cl2	2.304(2)	Cl2−Pt1−Cl1	91.6(5)
N7−C6	1.348(7)	C6−N7−Pt1	117.6(4)
N7−C8	1.337(7)	N7−C8−N1	128.1(5)
N1−C8	1.360(7)	N7−C8−C9	123.8(5)
N1−C2	1.382(8)	N1−C8−C9	108.1(5)
N1−C10	1.445(8)		

Table 2. Cytotoxicity [IC_{50} Mean Values (μM)] Obtained for 1, 2 and Cisplatin (72 h Incubation Time)[a]

	A2780	A2780cisR	MCF-7	HCT116 ($p53^{+/+}$)	HCT116 ($p53^{-/-}$)	Human primary skin fibroblasts
cisplatin	2.4 ± 0.1	15.6 ± 0.1 (6.5)	30 ± 1	5.9 ± 0.9	14 ± 2	69.0 ± 0.9
1	4.2 ± 0.2	4.3 ± 0.5 (1.0)	14 ± 2	10.2 ± 0.1	15 ± 1	51 ± 2
2	3.6 ± 0.1	2.8 ± 0.6 (0.8)	12 ± 2	5.2 ± 0.2	6.4 ± 0.1	17 ± 3

[a]The experiments were performed in triplicate. The results are expressed as mean values ± SD of three independent experiments, each of them made in quadruplicate. Resistance factor, defined as IC_{50}(resistant)/IC_{50}(sensitive), is given in parentheses.

Table 3. Cellular Accumulation of Platinum from 1, 2 and Cisplatin (at Equitoxic Concentrations) in A2780, A2780cisR, and MCF-7 Tumor Cells[a]

	A2780		A2780cisR		MCF-7	
	5 h	24 h	5 h	24 h	5 h	24 h
cisplatin	89 ± 1	181 ± 23	44 ± 5	128 ± 2	46 ± 11	100 ± 15
1	60.1 ± 0.4	139 ± 29	40 ± 4	126 ± 34	94 ± 15	298 ± 64
2	269 ± 35	595 ± 24	243 ± 27	640 ± 28	446 ± 23	893 ± 21

[a] Intracellular accumulation of **1**, **2** and cisplatin at equitoxic concentrations (IC_{50}) in A2780, A2780cisR and MCF-7 cells after 5 h or 24 h. Each value shown in the Table 3 is in pmoles of platinum per 10^6 cells. Results are expressed as the mean ± SD for three independent experiments.

Table 4. Platinum Content of DNA Isolated from A2780, A2780cisR or MCF-7 Cells Treated with Equitoxic Doses of 1, 2 or Cisplatin[a]

	A2780		A2780cisR		MCF-7	
	5 h	24 h	5 h	24 h	5 h	24 h
cisplatin	0.14 ± 0.03	0.34 ± 0.02	0.07 ± 0.02	0.10 ± 0.02	0.15 ± 0.01	0.24 ± 0.02
1	0.094 ± 0.005	0.39 ± 0.02	0.075 ± 0.002	0.31 ± 0.04	0.13 ± 0.02	0.43 ± 0.13
2	0.15 ± 0.02	0.51 ± 0.02	0.325 ± 0.002	0.94 ± 0.14	0.340 ± 0.003	0.82 ± 0.16

[a]Cells were exposed to tested compounds at equitoxic concentrations (IC_{50}) for 5 or 24 h. Each value shown in the Table 4 is in pmoles of Pt/μg of DNA. Results are expressed as the mean ± SD for three independent samples.

Table 5. Platinum Content of DNA Fraction Isolated from Cells Exposed to Equitoxic Doses of 1, 2 or Cisplatin Expressed as a Percentage of the Total Platinum Accumulated in the Cell[a]

	A2780		A2780cisR		MCF-7	
	5 h	24 h	5 h	24 h	5 h	24 h
cisplatin	1.1	1.3	1.1	0.6	2.3	1.7
1	1.1	1.9	1.3	1.7	1.0	1.0
2	0.4	0.6	0.8	1.0	0.5	0.6

[a]Cells were exposed to tested compounds at their equitoxic concentrations (IC_{50}) for 5 or 24 h.

Table 6. Crystal Data and Structure Refinement for *cis*-[$PtCl_2(NH_3)$(1M7AI)] (1).

Empirical formula	$C_8H_{11}Cl_2N_3Pt$
Formula weight	415.19
Crystal system	orthorhombic
Space group	*Pbca*
Unit cell dimensions (Å)	*a* = 10.1183(2)
	b = 10.4734(2)
	c = 21.2984(4)
Volume ($Å^3$)	2257.06(8)
Z	8
dimension (mm^3)	1.000 x 0.225 x 0140
Density (Mg/m^3)	2.444
Absorption coefficient (mm^{-1})	12.871
F(000)	1536
Theta range for data collection (°)	1.91 to 30.51
Index ranges	-14≤h≤14
	-14≤k≤14
	-30≤l≤30
Reflections collected	49459
Independent reflections	3447 [R(int) = 0.0280]
Data / restraints / parameters	3447 / 0 / 145
Goodness-of-fit on F^2	1.156
Final R indices [I>2sigma(I)]	R1 = 0.0301, wR2 = 0.0655

R indices (all data)	R1 = 0.0478, wR2 = 0.0760
Largest diff. peak and hole (e $Å^{-3}$)	2.385 and -1.064

Figure Captions

Figure 1. Sketch of the molecular structures of *cis*-[$PtCl_2(NH_3)$(1M7AI)] (**1**) and *trans*-[$PtCl_2(NH_3)$(1M7AI)] (**2**) used in this work; 1M7AI = 1-methyl-7-azaindole.

Figure 2. ORTEP drawing of a dimeric aggregate of *cis*-[$PtCl_2(NH_3)$(1M7AI)] (**1**) linked by hydrogen bonds.

Figure 3. Reaction of **1** (open symbols) or **2** (closed symbols) with (A) double-helical CT DNA ($NaClO_4$ 0.01 M) and (B) human serum albumin in Tris.HCl (10 mM, pH 7.4) plus NaCl (8 mM) at 310 K as a function of time.

Figure 4. RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment of pSP73KB plasmid modified by **1**, **2**, cisplatin or transplatin. A. Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel. Lanes: control, nonplatinated template; 1, 2, transPt, and cisPt, template modified by **1**, **2**, transplatin or cisplatin, respectively, at $r_b = 0.01$; C, G, U, and A, chain terminated markers for nonplatinated template. B. Sequence of the NdeI/HpaI fragment of the pSP73KB plasmid. The arrow indicates the start of T7 RNA polymerase. Numbers correspond to nucleotide numbering in the sequence of the pSP73KB plasmid. Circles and squares indicate stop signals from panel A, lanes **1** and **2**, respectively.

Figure 5. Top panels (A-C): Confocal microscope images of A2780 cells exposed to 30 μM **1** (A) or **2** (B) for 5 h or which were untreated (C). Scale bars represent 10 μm.

Bottom panels (D,E): Confocal microscope images of A2780 cells treated with 30 μM of **1** (panels D), **2** (panels E) or 1-methyl-7-azaindole (panels F) for the time indicated in each panel. Scale bars represent 25 μm.

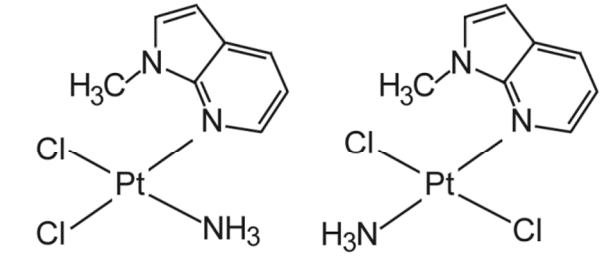
Figure 6. Effects of **1**, **2**, and cisplatin on cell cycle distribution. Untreated (control) A2780 cells or A2780 cells treated with equitoxic concentration of **1** (4.2 μM), **2** (3.6 μM) or cisplatin (2.4 μM) for 24 h (left panel) or 72 h (right panel) were harvested, fixed, stained with propidium iodide, and assessed for cell cycle distribution by fluorescence-activated cell sorting (FACS). The estimated percentages of A2780 cells in different phases of the cell cycle are indicated. The results are expressed as the mean ± the standard deviation (SD) of three independent experiments. The symbol (*) denotes a significant difference ($p < 0.05$) from the untreated control; (#) denotes a significant difference ($p < 0.05$) between **1** or **2** and cisplatin.

Figure 7. Effects of **1**, **2** and cisplatin on activation of the apoptotic and necrotic pathways in A2780 cells determined by DNA fragmentation ELISA assays. A. Cells treated for 24 h with **1**, **2** or cisplatin at the equitoxic concentrations indicated in the figure. Left panel, apoptosis; right panel, necrosis. B. Cells treated for 72 h with **1**, **2** or cisplatin at a concentration corresponding to the IC_{50} value (Table 2). The results are expressed as the mean of two independent experiments with duplicate runs. The levels of DNA fragmentation found for control, untreated, cells were subtracted from each column.

Figure 8. Comparison of IC_{50} values determined in A2780 cells for complexes **1**, **2** or cisplatin after 24 or 72 h treatment. The results are expressed as the mean ± SD of two independent experiments with quadruplicate runs.

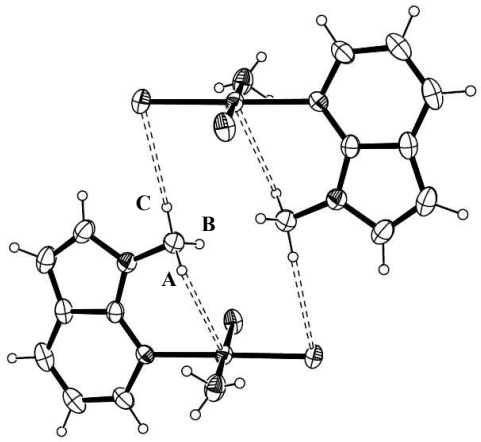
Figure 9. Sensitivity of HCT116 cells [parental cells ($p53^{+/+}$) and its p53-null derivative ($p53^{-/-}$)] to the treatment with **1**, **2** and cisplatin. DNA quantification of total cells after treatment with **1** (A), **2** (B) and cisplatin (C) assessed as a measure of fluorescence of Sybr Green I. DNA contents of control, untreated cells were taken as 100%. The error bars represent the SDs, experiments were done in triplicate. (*) denotes significant ($p<0.05$) difference from p53-wild type ($p53^{+/+}$) cells.

Figure 1



cis-[$PtCl_2(NH_3)$(1M7AI)] *trans*-[$PtCl_2(NH_3)$(1M7AI)]

Figure 2



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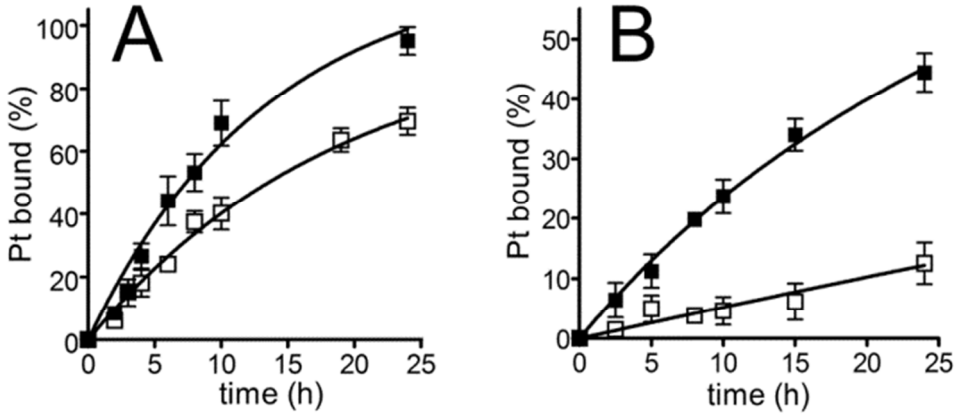

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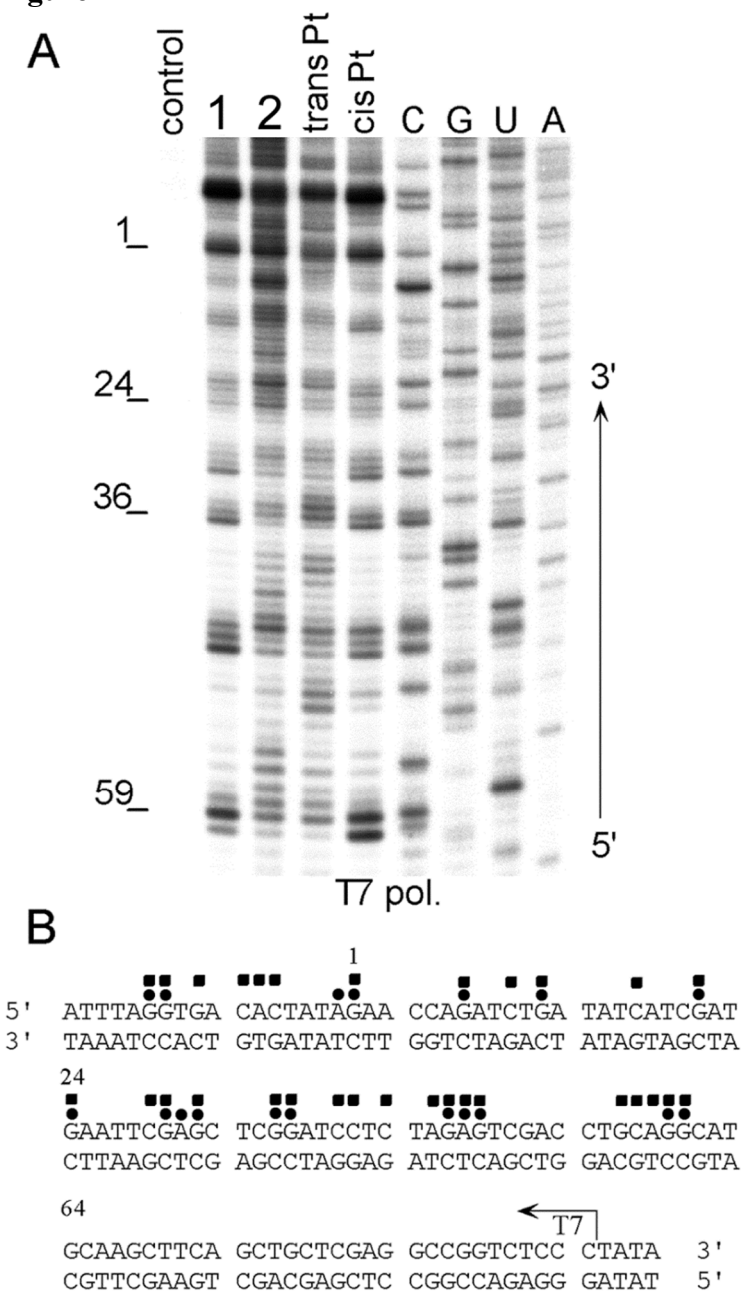
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control
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2
trans Pt
cis Pt
C
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U
A
1
24
36
59
3'
5'
T7 pol.
B
1
5' ATTTAGGTGA CACTATAGAA CCAGATCTGA TATCATCGAT
3' TAAATCCACT GTGATATCTT GGTCTAGACT ATAGTAGCTA
24
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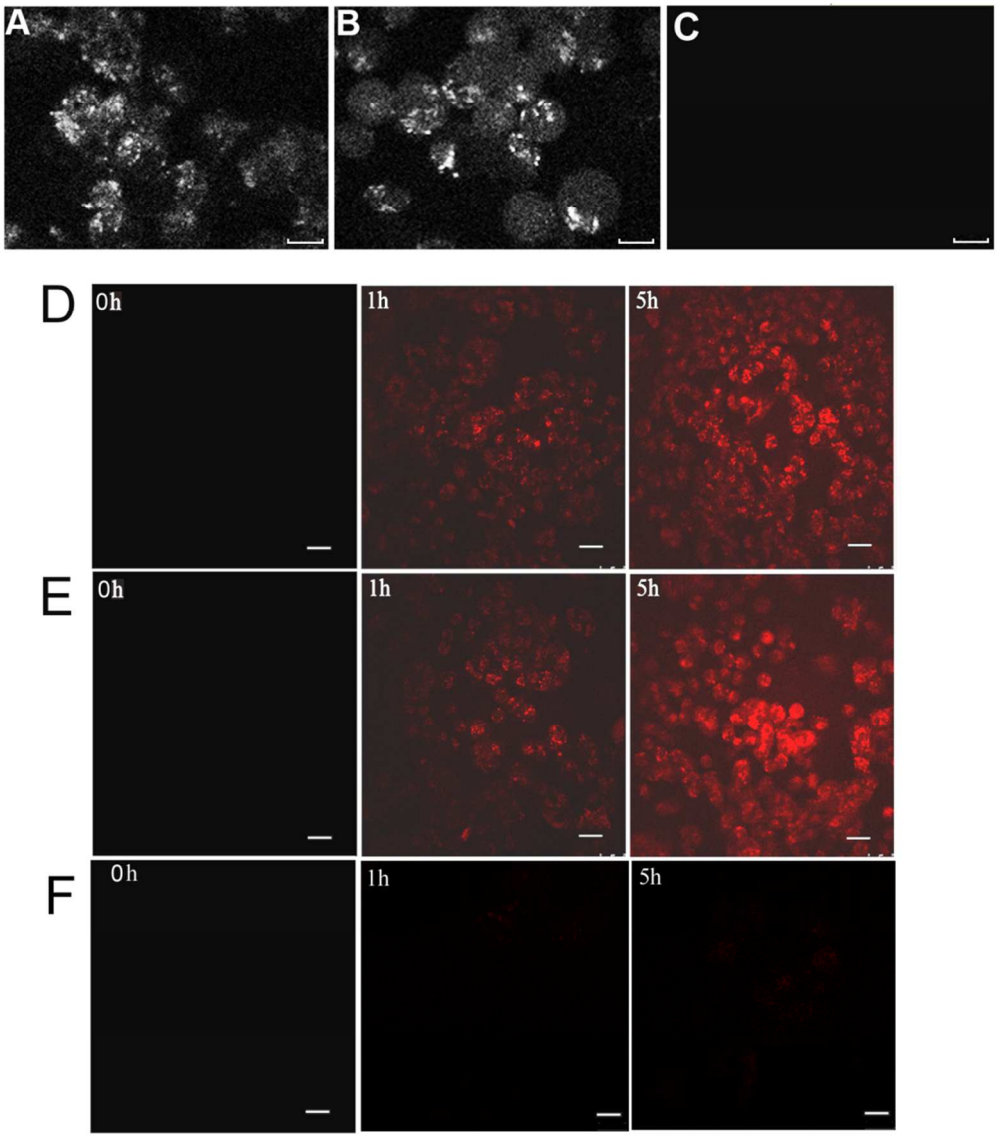
Figure 5



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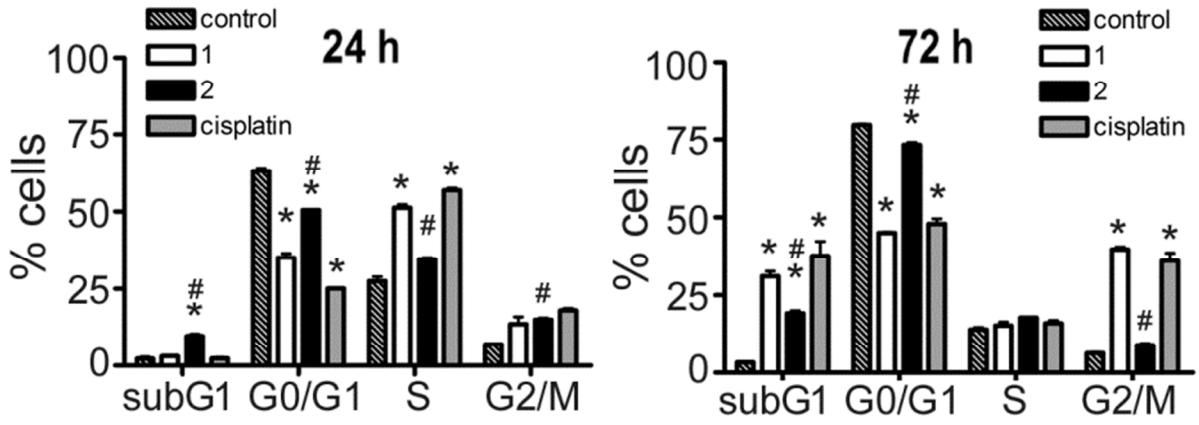

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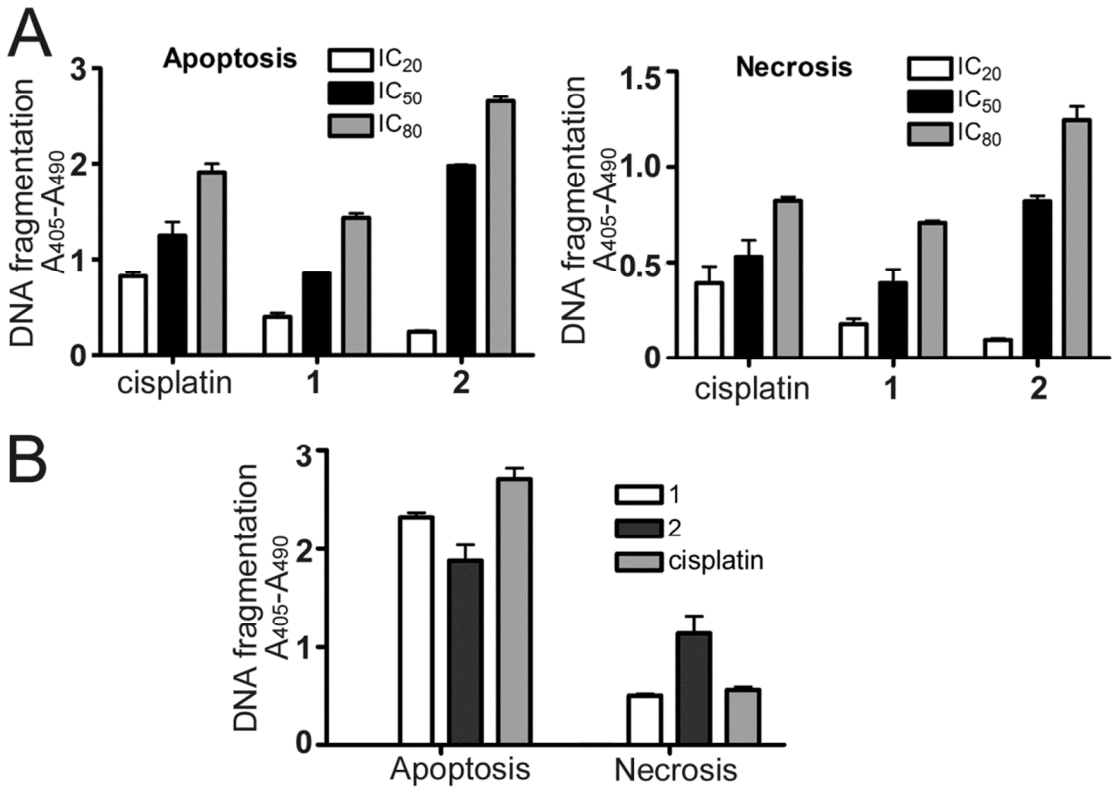


Figure 8

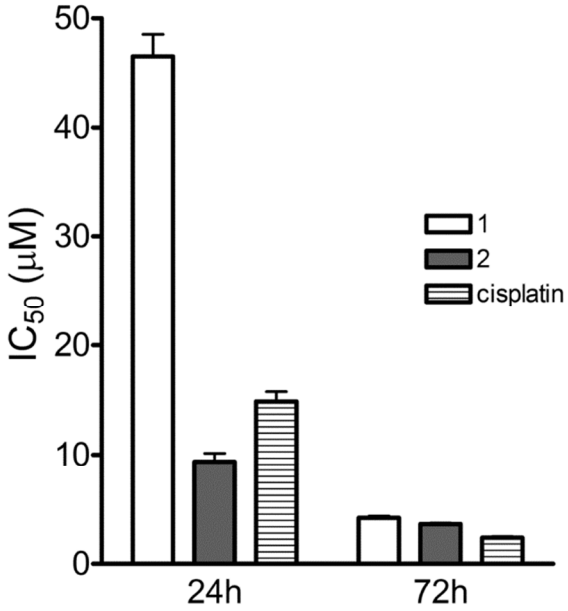
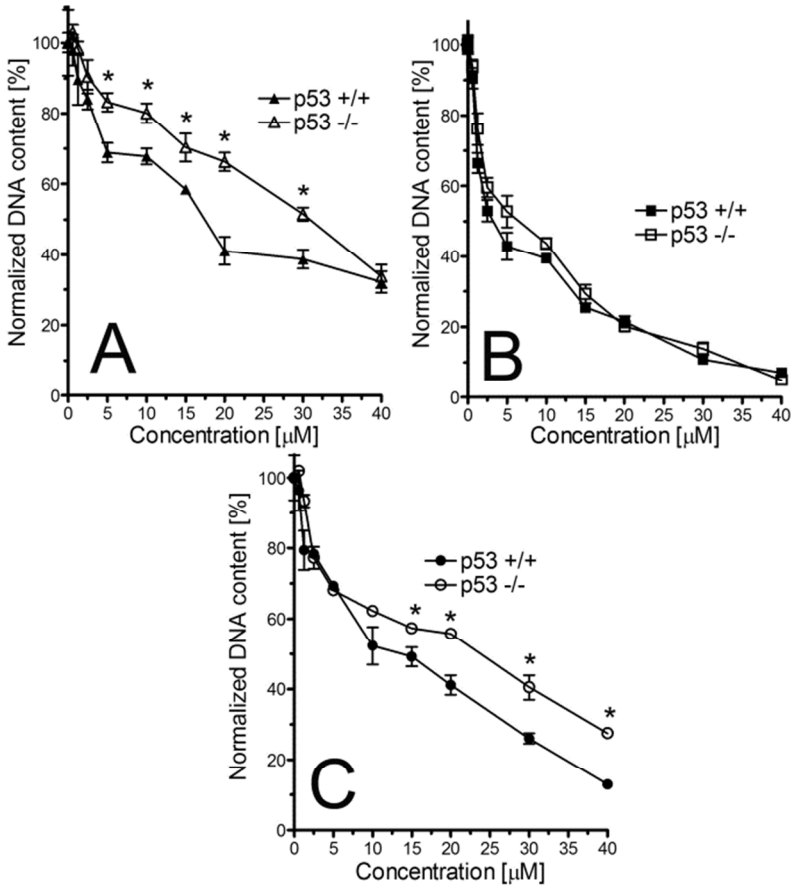


Figure 9



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