Novel Apoptosis-Inducing *trans*-Platinum Piperidine Derivatives: Synthesis and Biological Characterization

Elena Khazanov,[†] Yechezkel Barenholz,[†] Dan Gibson,*[,‡,§] and Yousef Najajreh[‡]

Laboratory of Membrane and Liposome Research, Department of Biochemistry, The Hebrew University−Hadassah Medical School, P. O. Box 12272, Jerusalem 91120, Israel, and Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University, P. O. Box 12065, Jerusalem 91120, Israel

Received January 9, 2002

The synthesis, chemical characterization, and interaction with cells of new sterically hindered *trans*- and *cis*-diaminedichloroplatinum(II) complexes are described. The amine ligands include monofunctional piperidine (pip) and piperazine (pz). The poor solubility of *trans*-diaminedichloroplatinum complexes was overcome by introducing the positively charged pz ligand, which allows retaining of the classic platinum coordination sphere. In vitro evaluation in OV-1063 and C-26 tumor cells revealed that replacing one NH_3 of the inactive transplatin by an aromatic planar ligand (4-picoline, 4-pic) or by an aliphatic nonplanar heterocyclic ligand (pip) or replacing both NH_3 groups with a 4-pic ligand and a pip or pz ligand significantly increases the cytotoxic activity of these complexes. The unsymmetric complexes *trans*-[$PtCl_2$(4-pic)(pip)] and *trans*-[$PtCl_2$(4-pic)(pz)]HCl were the most cytotoxic compounds against the cisplatin-sensitive tumor cell line C-26 (IC_{50} = 4.5 and 5.5 μM, respectively) and the cisplatin-sensitive tumor cell line OV-1063 (IC_{50} = 6.5 and 7.4 μM, respectively). In contrast, replacing one NH_3 of the cis isomer by an aromatic planar ligand (4-pic) or by an aliphatic amine lowered their cytotoxiciy in comparison to cisplatin. Cell penetration and Pt−DNA adduct formation were also evaluated, and it was clearly shown that both *trans*-[$PtCl_2$(4-pic)(pip)] and *trans*-[$PtCl_2$(4-pic)(pz)]HCl penetrate efficiently the cellular membrane of the tumor cells and platinate the cellular DNA. When comparing cellular DNA platination, positively charged *trans*-[$PtCl_2$(4-pic)(pz)]HCl was 7-fold higher than both cisplatin and its neutral analogue *trans*-[$PtCl_2$(4-pic)(pip)]. Moreover, in contrast to cisplatin, in the cell lines used, cell death caused by both complexes appeared to be apoptotic according to several criteria including early phosphatidylserine exposure, activation of caspases, and characteristic morphological changes. Our results suggest that these novel mixed nonclassical *trans*-Pt(II) complexes are biologically and mechanistically distinct from known Pt complexes and deserve evaluation of their efficacy in tumor-bearing animals.

Introduction

Cisplatin, *cis*-[$PtCl_2(NH_3)_2$] (Figure 1a), is one of the most widely used clinical agents in the treatment of a variety of solid tumors.[1,2] It is believed to kill tumor cells by binding irreversibly to the DNA, mainly to two adjacent guanines on the same strand, inducing a kink in the DNA that is recognized by cellular proteins that bind the cisplatin-modified DNA.[3] It is the Pt−DNA adducts that are responsible for the induction of cell death.[4] Despite its efficacy in the treatment of various neoplastic diseases, including testicular and ovarian tumors, its clinical utility is restricted by its low solubility, toxicity, and, especially, by inherent and acquired tumor resistance.[5] Second-generation drugs such as carboplatin (Figure 1b) exhibit reduced nephrotoxicity but fail to overcome the tumor resistance, probably due to the fact that they form the same spectrum of DNA adducts as does cisplatin. Overcoming the resistance is one of the major goals in the development of novel platinum drugs; hence, new compounds that deviate from the classic structure−activity relationships (SAR) have been designed, synthesized, and

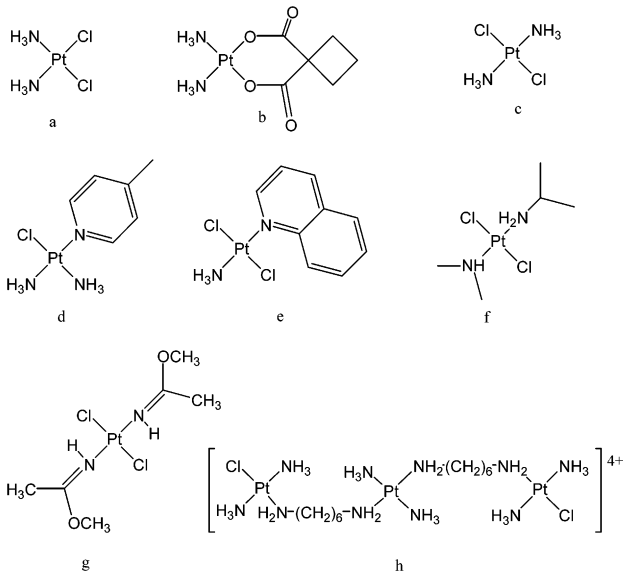


Figure 1. Platinum anticancer agents (a) cisplatin, (b) carboplatin, (c) transplatin (not active), (d) *cis*-$Pt(NH_3)_2$(4-pic)-Cl, (e) *trans*-$PtCl_2(NH_3)$(quin), (f) *trans*-$PtCl_2$(ipa)(dma), (g) *trans*-$PtCl_2(EE)_2$, and (h) BBR3464.

* To whom correspondence should be addressed. Tel: (972)2-6758702. Fax: (972)2-6757076. E-mail: gibson@md2.huji.ac.il.
[†] The Hebrew University−Hadassah Medical School.
[‡] School of Pharmacy.
[§] Affiliated with the David R. Bloom Center for Pharmacy at The Hebrew University of Jerusalem.

10.1021/jm020817y CCC: $22.00
Published on Web 10/30/2002

screened.[6,7] The SAR, first formulated by Cleare and Hoeschele, influenced medicinal chemists to direct their efforts to the preparation of neutral platinum(II) complexes with two inert ligands in the cis configuration and two semilabile leaving groups.[8]

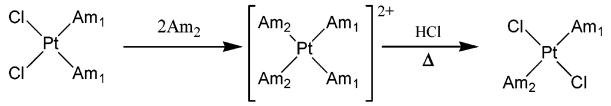
There is a growing number of nonclassical platinum compounds that possess favorable therapeutic properties. Hollis et al. reported that positively charged triaminemonochloroplatinum(II) complexes of the general formula *cis*-$[Pt(NH_3)_2(Am)Cl]Cl$ (Figure 1d is an example for Am = 4-picoline (pic)) have cytotoxic activity.[9,10] These complexes violated two basic rules: they had three inert ligands and one semilabile, and they were positively charged. Farrell et al. reported on the activation of the trans geometry with planar amine ligands of the type *trans*-$[PtCl_2(L)(L')]$ where L = NH_3 and L′ = quinoline, thiazole, etc. (Figure 1e is an example where L′ = quinoline).[11,12] This discovery by Farrell led to the development of the field of trans platinum compounds as potential anticancer agents. Navarro-Ranninger and co-workers demonstrated that *trans*-$\{[PtCl_2[NH_2CH(CH_3)_2][NH(CH_3)_2]\}$ (Figure 1f) has interesting pharmacological properties,[13,14] and Natile and co-workers reported that *trans*-$[PtCl_2(iminoether)_2]$ (Figure 1g) is also active against several human cancer lines.[15] Another example of a nonclassical complex, which is now in phase 2 of clinical trials, is the trinuclear Pt complex BBR3464, which has a quadruply charged cation (Figure 1g).[16] The importance of the nonclassical platinum compounds stems from the fact that they were designed to form a spectrum of DNA adducts that is distinct from that formed by cisplatin and carboplatin; hence, they can circumvent acquired cisplatin resistance.[17,18]

Generally, *trans*-diaminedichloroplatinum(II) analogues have lower solubility in aqueous solution than their cis counterparts, resulting in limited bioavailability. One way of increasing the aqueous solubility is by adding a charge to the complex. The cationic charges of the platinum complexes prepared by Hollis and by Farrell reside on the metal center and result from the substitution of one of the anionic chloride ligands by a neutral ligand. These complexes do not have the "classic" diaminedichloro coordination sphere.

The ability of certain antitumor drugs to achieve a significant therapeutic index differentiating malignant from normal cells may be associated with tumor cell death through induction of apoptosis at drug concentrations significantly lower than those needed to kill normal cells.[19] Several platinum complexes have been reported to induce apoptosis in cancer cells. Cisplatin and *cis*- and *trans*-amine(cyclohexylamine)dihydroxodichloroplatinum(IV) have been shown to kill CH1 human ovarian carcinoma cells by induction of apoptosis, and more recently, *trans*-$[PtCl_2(amine)(isopropylamine)]$ was reported to induce apoptosis in *ras*-transformed cells.[20,21] Thus, novel trans complexes should be tested for their ability to induce apoptosis.

In this paper, we describe the synthesis, characterization, and pharmacological properties of a class of novel neutral compounds and one charged *trans*-diaminedichloroplatinum(II) compound where at least one of the inert amine ligands is a nonplanar heterocyclic amine such as piperidine (pip) or piperazine (pz). The results of in vitro cytotoxic activity against C-26 and OV-1063 cells are reported, as well as direct interaction with DNA, cell uptake, levels of cellular DNA platination, and the ability of the complexes to induce apoptosis.

Scheme 1



Results

Synthesis and Chemical Characterization. The general procedure for the synthesis of the unsymmetric *trans*-diaminedichloroplatinum(II) complexes is depicted in Scheme 1. Initially, the symmetric *cis*-$[PtCl_2(Am_1)_2]$ was prepared by direct reaction of 2 equiv of the amine (Am_1) with tetrachloroplatinate (K_2PtCl_4). The neutral products precipitated in high yields from the aqueous solution and were characterized by ^{195}Pt NMR spectroscopy. The *cis*-$[PtCl_2(Am_1)_2]$ complexes were further reacted with 2 equiv of the second amine (Am_2) to yield the cis-oriented, doubly charged tetraamine complex *cis*-$[Pt(Am_1)_2(Am_2)_2]^{2+}$. The tetraamine complexes were treated with hydrochloric acid to yield the desired *trans*-$[PtCl_2(Am_1)Am_2)]$ complexes. Compounds **I**−**III** were prepared in this fashion in good yields. The final products were characterized by ^{195}Pt NMR spectroscopy and by electrospray ionization mass spectrometry (ESI MS), and their purity was ascertained by elemental analysis.

The synthesis of unsymmetric *cis*-$[PtCl_2(NH_3)(Am)]$ can be accomplished in one of two ways: the preparation of $[PtCl_3(NH_3)]^-$ and the subsequent reaction with Am or by treatment of $[PtI_2(Am_2)]_2$ with perchloric acid and NH_3 to obtain the *cis*-$[PtI_2(NH_3)(Am_2)]$, which is converted to *cis*-$[PtCl_2(NH_3)(Am_2)]$ by treatment with $AgNO_3$ and subsequently with KCl. Attempts to prepare the pip complex using the former procedure failed, probably due to the strong basicity of the pip. Thus, the latter procedure was followed and yielded the desired products in reasonable yields.

In Vitro Growth Inhibition. To assess the cytotoxic activity of complexes (**I**−**VI**) against C-26 and OV-1063 cancer cell lines, they were incubated for 4, 24, or 72 h with the cells. Methylene blue (MB) cytotoxicity assays revealed that replacing one or both ammine ligands of transplatin enhanced significantly (by more than 4-fold) the cytotoxicity of the new *trans*-$[PtCl_2(Am_1)(Am_2)]$ compounds **I**−**IV** in both C-26 and OV-1063 cell lines (Table 1). Replacing one NH_3 by either an aromatic planar amine (4-pic) to give *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$ (**II**) or by an aliphatic nonplanar heterocyclic amine (pip) to give *trans*-$[PtCl_2(NH_3)(pip)]$ (**III**) enhances the cytotoxic activity relative to transplatin (Table 1). Interestingly, the mixed *trans*-$[PtCl_2(4\text{-pic})(pip)]$ (**I**) is more cytotoxic than **II** or **III** by a factor of 2−3 (Table 1) and is 3-fold less active than cisplatin.

The cytotoxicity of the sterically hindered trans compounds was compared with that of their cis counterparts—*cis*-$[PtCl_2(NH_3)(4\text{-pic})]$ (**V**) and *cis*-$[PtCl_2(NH_3)(pip)]$ (**VI**). In contrast to the *trans*-Pt complexes, replacement of one NH_3 of cisplatin by 4-pic or by pip resulted in lower cytotoxic activity as compared to cisplatin. *cis*-$[PtCl_2(NH_3)(pip)]$ is an analogue of the new

Table 1. IC_{50} of Complexes **I**−**VI** against C-26 and OV-1063 Cells as Compared to Cisplatin and Transplatin[a]

		C-26 cells			OV-1063 cells		
	complex no.	IC_{50} (μM) 4 h	IC_{50} (μM) 24 h	IC_{50} (μM) 72 h	IC_{50} (μM) 4 h	IC_{50} (μM) 24 h	IC_{50} (μM) 72 h
cisplatin		1.5 ± 1.3	0.6 ± 0.1	0.2± 0.2	2.0 ± 0.5	0.7 ± 0.2	0.5 ± 0.2
transplatin		64.0 ± 2.0	46.0 ± 3.1	41.0 ± 1.7	81.0 ± 4.3	73.0 ± 5.1	52.0 ± 3.2
trans-[$PtCl_2$(4-pic)(pip)]	**I**	4.5 ± 0.7	2.5 ± 0.7	1.75 ± 1.0	6.5 ± 0.7	6.0 ± 1.4	4.5 ± 2.1
trans-[$PtCl_2(NH_3)$(4-pic)]	**II**	12.0 ±1.2	11.0± 2.0	11.0 ± 1.1	18.0 ± 3.7	16.0 ± 3.5	14.0 ± 2.3
trans-[$PtCl_2(NH_3)$(pip)]	**III**	8.5 ± 2.0	7.7 ± 2.0	7.0 ± 2.5	11.0 ± 2.0	9.5 ± 1.7	8.7 ± 1.4
trans-[$PtCl_2$(4-pic)(pz)]HCl	**IV**	5.5 ± 1.0	4.5 ± 0.75	3.5 ± 0.8	7.4 ± 1.5	6.0 ± 1.0	5.1 ± 0.9
cis-[$PtCl_2(NH_3)$(4-pic)]	**V**	5.1 ± 1.2	4.7 ± 2.4	3.7 ± 1.6	6.0 ± 2.2	5.1 ± 1.9	4.0 ± 1.7
cis-[$PtCl_2(NH_3)$(pip)]	**VI**	2.6 ± 1.7	2.1 ± 1.2	1.3 ± 0.7	4.2 ± 1.7	3.1 ± 1.2	2.6 ± 0.7

[a] Mean ± SD from at least two experiments.

Table 2. Drug Solubility in Aqueous Solution

drugs	solubility in DDW mg/mL (mM), 37 °C
trans-[$PtCl_2$(4-pic)(pip)] (**I**)	0.031 (0.07)
trans-[$PtCl_2(NH_3)$(4-pic)] (**II**)	0.30 (0.79)
trans-[$PtCl_2(NH_3)$(pip)] (**III**)	0.30 (0.81)
trans-[$PtCl_2$(4-pic)(pz)]HCl (**IV**)	7.5 (18.0)
cis-[$PtCl_2(NH_3)$(4-pic)] (**V**)	0.2 (0,53)
cis-[$PtCl_2(NH_3)$(pip)] (**VI**)	0.75 (2.0)
transplatin	0.24 (0.8)
cisplatin	2.1 (6.3)

active *cis*-[$Pt(NH_3)$(2-pic)] (AMD0473), a novel sterically hindered antitumor compound designed to circumvent platinum drug resistance, which is currently undergoing clinical trials.[16]

The poor solubility of the neutral trans compounds prompted us to synthesize the positively charged analogue *trans*-[$PtCl_2$(4-pic)(pz)·HCl] (**IV**), which is much more water soluble than compound **I** (Table 2). Compound **IV** has the same cytotoxic activity as compound **I** against both cell lines.

The cytotoxicity data presented here show that the *trans*-[$PtCl_2$(4-pic)(pip)] and *trans*-[$PtCl_2$(4-pic)(pz)·HCl] are the most cytotoxic *trans*-Pt complexes in both cell lines. To our knowledge, compounds **I** and **IV** are the first examples of diaminedichloroplatinum(II) compounds with one aromatic amine ligand and one heterocyclic aliphatic amine ligand that possess cytotoxic activity in ovarian and colon cancer cell lines.

Cellular Drug Uptake and DNA Platination. To determine drug accumulation in tumor cells, the C-26 and OV-1063 cells were exposed to the most cytotoxic compounds, *trans*-[$PtCl_2$(4-pic)(pip)] and *trans*-[$PtCl_2$-(4-pic)(pz)·HCl], for 24 h and compared with the drug uptake of cisplatin and transplatin under the identical conditions. The Pt content associated with the cells was measured by atomic absorption spectroscopy (AAS). *trans*-[$PtCl_2$(4-pic)(pip)] penetrates the cells very efficiently in both cell lines. It penetrates OV-1063 and C-26 cells 7-fold and 30-fold, respectively, more efficiently than transplatin (Figure 3b,a respectively). The *trans*-[$PtCl_2$(4-pic)(pz)]HCl has the highest penetration values in both cell lines (Figure 3a,b). A time-dependent increase of *trans*-[$PtCl_2$(4-pic) (pip)] and *trans*-[$PtCl_2$(4-pic)(pz)]HCl accumulation was observed from 4 (data not shown) to 24 h of drug exposure.

To determine the platination level of cellular DNA, C-26 and OV-1063 cells were exposed to complexes **I** and **IV** for 4 (data not shown) or 24 h and compared with cisplatin (Figure 4a,b, respectively). The platinum content was measured by AAS. The DNA platination level

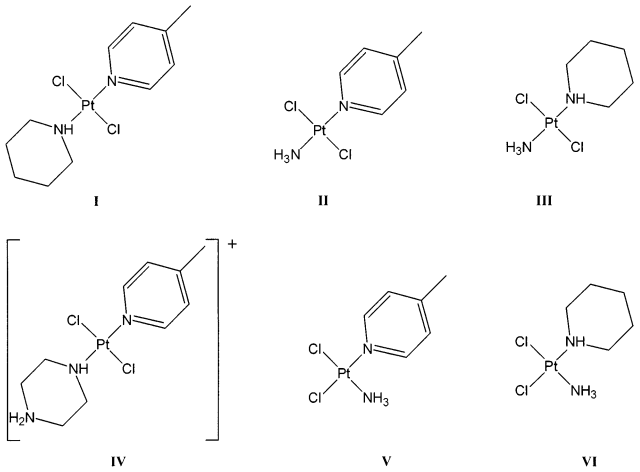


Figure 2. Piperazine and pip complexes used in this study.

with *trans*-[$PtCl_2$(4-pic)(pip)] was the same as that with cisplatin in C-26 and OV-1063 cells (Figure 4a,b respectively). The values of Pt species bound to DNA in the case of *trans*-[$PtCl_2$(4-pic)(pz)]HCl were 7-fold higher than of cisplatin adducts in both cell lines (Figure 4a,b respectively).

Assessment of Apoptosis. Apoptosis, or programmed cell death, is involved in the regulation of cell number in multicellular organisms and the pathogenesis of various diseases, including tumor progression, neurodegenerative disorders, and viral infections.[30] Externalization of phosphatidylserine (PS) as well as condensation of nucleoplasm with a pronounced decrease of cell volume and condensation of chromatin were evaluated by combined MC 540 and DAPI staining of treated cells. In most cell types, PS, a lipid normally confined to the inner leaflet of the plasma membrane, is exported to the outer plasma membrane leaflet in the early stage of apoptosis.[31] PS exposure in treated C-26 and OV-1063 cells was detected by staining with MC 540, which has a strong affinity to PS. Chromatin condensation was assessed by staining with DAPI, which preferentially stains dsDNA. Figure 5b shows distinct features of apoptosis in *trans*-[$PtCl_2$(4-pic)(pip)]-treated OV-1063 cells. This is evidenced by the appearance of red fluorescence in the cell membrane, nucleoplasm and cytoplasm condensation with a pronounced decrease of cell volume, and enhanced green fluorescence in the nucleus, in contrast with the untreated cells (Figure 5a). The results of this staining show that a large proportion of the OV-1063 cells appeared to be apoptotic after 5 h of treatment with 6.5 μM *trans*-

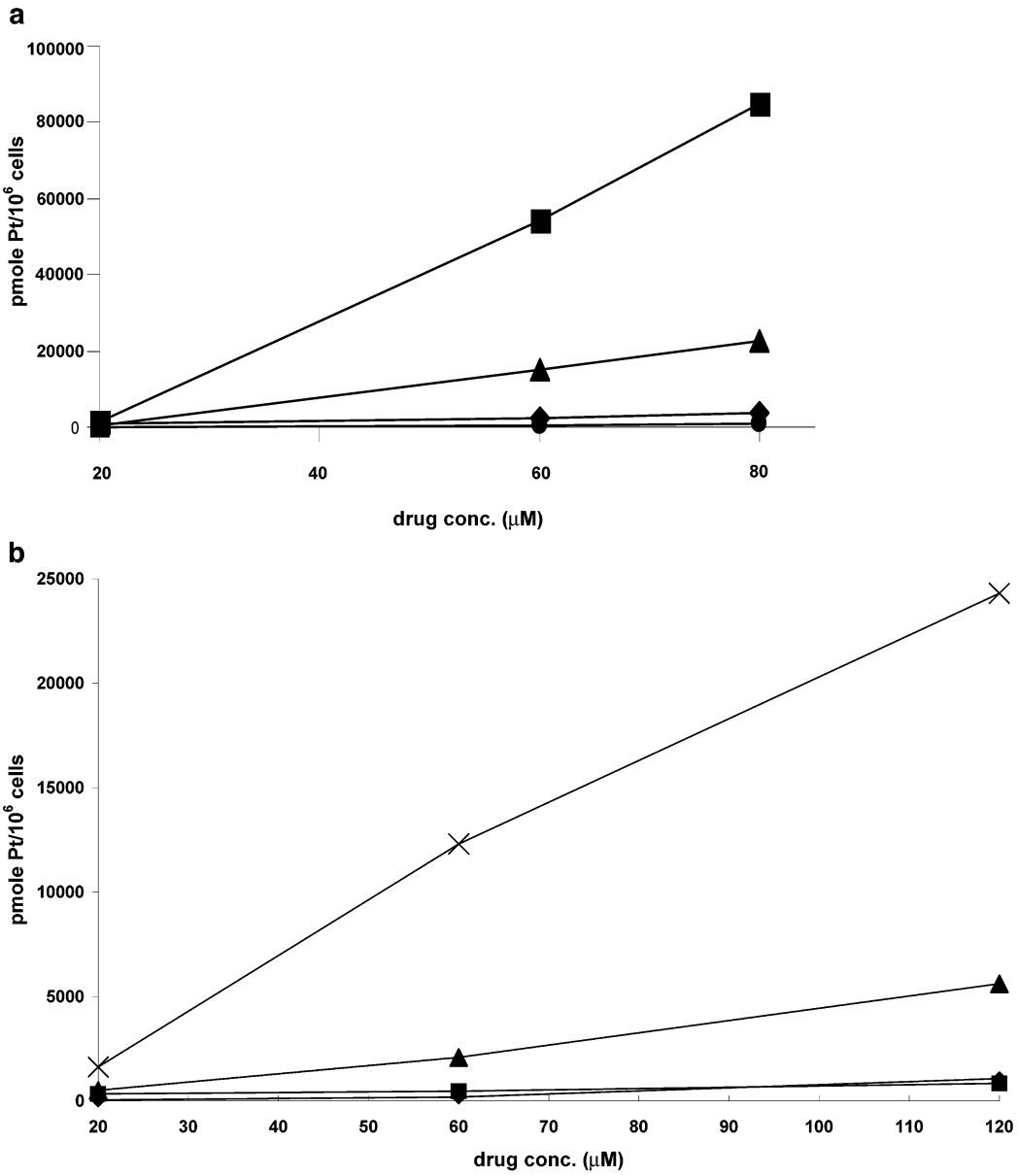


Figure 3. Cellular accumulation of platinum (by AAS) in C-26 cells (a) or OV-1063 cells (b) treated with cisplatin (filled diamonds), transplatin (filled circles), *trans*-[$PtCl_2$(4-pic)(pip)] (filled triangles), or *trans*-[$PtCl_2$(4-pic) (pz)]HCl (×) for 24 h. Data represent the mean value of two independent experiments.

[$PtCl_2$(4-pic)(pip)]. The cell surface of C-26 cells became slightly red fluorescent after 5 h of treatment with 4.5 μM *trans*-[$PtCl_2$(4-pic)(pip)] (Figure 5d) in contrast with the lack of fluorescence in the untreated cells (Figure 5c).

Recently, members of the caspase (CED-3/ICE) family of proteases have been found to be crucial mediators of the complex biochemical events associated with apoptosis.[32] In particular, the activation of caspase-3, which cleaves a number of different proteins, including poly(ADP-ribose) polymerase (PARP), protein kinase Cδ, and actin, has been shown to be important for the initiation of apoptosis.[33] The activation of caspase-3 was measured in *trans*-[$PtCl_2$(4-pic)(pip)]- or in *trans*-[$PtCl_2$-(4-pic)(pz)]HCl-treated C-26 and OV-1063 cells. OV-1063 cells that were treated for 5 h with 6.5 μM *trans*-[$PtCl_2$-(4-pic)(pip)] or with 7.5 μM *trans*-[$PtCl_2$(4-pic)(pz)]HCl revealed activation of caspase-3 ($\sim$2-fold increase in fluorescence in treated cells in comparison to untreated cells) (Figure 6). Moreover, after 16 h of treatment of OV-1063 cells with *trans*-[$PtCl_2$(4-pic)(pip)] or with *trans*-[$PtCl_2$(4-pic)(pz)]HCl, there was $\sim$3-fold increase in fluorescence in treated cells in comparison to untreated cells (Figure 6). To confirm that the observed fluorescent signal was due to activation of caspase-3, the reversible Ac-DEVD-CHO inhibitor of caspase-3-like proteases was added to the control and treated samples. A drastic decrease in fluorescent signal was found in drug-treated samples after addition of Ac-DEVD-CHO inhibitor (data not shown), which argues for specific activation of caspase-3. No fluorescent signal was observed in C-26 cells treated with IC_{50} values of $PtCl_2$-(4-pic)(pip)] or *trans*-[$PtCl_2$(4-pic)(pz)]HCl (data not shown).

To determine whether OV-1063 or C-26 cells treated with cisplatin undergo apoptosis, these cell lines were treated for 5 or 16 h with 2 or 1.5 μM cisplatin, respectively. No fluorescent signal was observed in cisplatin-treated OV-1063 cells (Figure 6) or in C-26 cells. These findings are in agreement with the data of

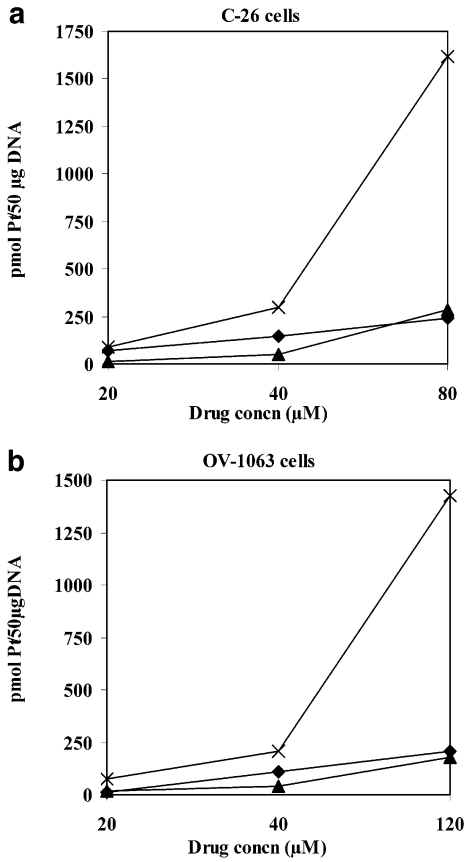


Figure 4. Cellular DNA platination (by AAS) in C-26 cells (a) or OV-1063 cells (b) treated with cisplatin (filled diamonds), *trans*-[$PtCl_2$(4-pic)(pip)] (filled triangles), or *trans*-[$PtCl_2$(4-pic)(pz)]HCl (×) for 24 h. Data represent the mean value of two independent experiments.

Szmigiero and co-workers,[34] which demonstrated that there was no degraded DNA detected by agarose gel electrophoresis in L1210 cells treated with cisplatin. It is also in agreement with findings, that have shown that colon cancer cells protect themselves from apoptosis by secreting soluble factor(s)[35] and by aberrant activation of c-kit.[36]

Study of the DNA Adducts by EtBr Fluorescence. EtBr, as a fluorescent probe, can be used to distinguish between perturbations induced in DNA by adducts of platinum(II) compounds.[28] Binding of EtBr by intercalation is blocked in a stoichiometric manner by formation of the bifunctional adducts, as of cisplatin, which results in a loss of fluorescence intensity.[28] On the other hand, formation of monofunctional adducts results only in a slight decrease of EtBr fluorescence. DNA modified by *trans*-[$PtCl_2$(4-pic)(pz)]HCl shows a considerable decrease in fluorescence (similar to cisplatin), which indicates loss of some double helical structure, probably due to the formation of bifunctional adducts (Figure 7). The decrease of fluorescence intensity by the adducts of *trans*-[$PtCl_2$(4-pic)(pip)] was smaller than that of cisplatin but greater than that of transplatin (Figure 7).

Discussion

Several new structural types of biologically active *trans*-dichlorodiamine−platinum complexes have been identified in recent years. The amine ligands include planar aromatic amines, linear and cyclic aliphatic amines, and iminoethers. While heterocyclic nonplanar amines such as pip and pz were used as monofuctional or chelating ligands, respectively, for *cis*-$PtCl_2$ and for Pt(malonate), to the best of our knowledge, there are no reports on the use of these ligands in SAR studies of *trans*-platinum complexes. The pip and pz ligands are bulkier than their planar pyridine analogues but not as bulky as their noncyclic secondary amine counterparts. They do have conformational flexibility (boat and chair) that provides them with some freedom to optimize their interactions with DNA; they cannot however intercalate like their planar analogues. The platinum can bind to the heterocyclic amine either in the axial or in the equatorial position. The X-ray crystal structure of complex **I** shows that the platinum is bound to the equatorial position and the six-membered ring is in the chair conformation (results not shown). The pz ligand has two features that could be important in its interactions with DNA. It is positively charged under physiological conditions, and it can form hydrogen bonds through the amine in position 4. These structural features could facilitate novel types of lesions with cellular DNA.

Cisplatin is believed to enter the cell by passive diffusion, and the earlier studies implied that charged platinum complexes could not efficiently enter cancer cells. Hollis and Farrell were the first to demonstrate that cationic platinum compounds are cytotoxic and therefore are efficiently internalized by cells. The triplatinum complex BBR3464, which is currently in phase 2 clinical trials, is quadruply charged (4+), yet efficiently enters cells and forms novel types of adducts with DNA.[37] Our results indicate that the positively charged *trans*-[$PtCl_2$(4-pic)(pz)]$^+$ is internalized by colon cancer cells and by ovarian cancer cells four times more efficiently than its neutral *trans*-[$PtCl_2$(4-pic)(pip)] analogue. Moreover, the charged *trans*-[$PtCl_2$(4-pic)(pz)]$^+$ complex is also much more efficient in platinating DNA than the corresponding neutral pip complex. In summary, we found a positive correlation between solubility of tested complexes, their intracellular penetration, and formation of Pt−DNA adducts.

Cisplatin, *trans*-[$PtCl_2$(4-pic)(pz)]HCl, and *trans*-[$PtCl_2$-(4-pic)(pip)] were reacted with calf thymus DNA, and the half-lives ($t_{1/2}$) for these reactions were 100, 12, and 113 min, respectively (unpublished results). These results are in agreement with the observations described in this study, leading us to conclude that adding a positive charge and/or a hydrogen bond donor to the ligand significantly increases the rate of DNA binding.[37]

Although cytotoxicity is associated with formation of platinum−DNA adducts, it seems that for these complexes there is no direct correlation between the levels of DNA platination and the in vitro cytotoxicity. Comparison of the DNA platination levels in both cell lines shows that the order of efficiency of DNA platination is *trans*-[$PtCl_2$(4-pic)(pz)]HCl ≫ *trans*-[$PtCl_2$(4-pic)(pip)] > cisplatin, while the relative cytotoxicity of these complexes is cisplatin > *trans*-[$PtCl_2$(4-pic)(pip)] > *trans*-[$PtCl_2$(4-pic)(pz)]HCl. DNA platination is a necessary condition for cytotoxic activity of platinum complexes but not a sufficient one. Other platinum complexes such

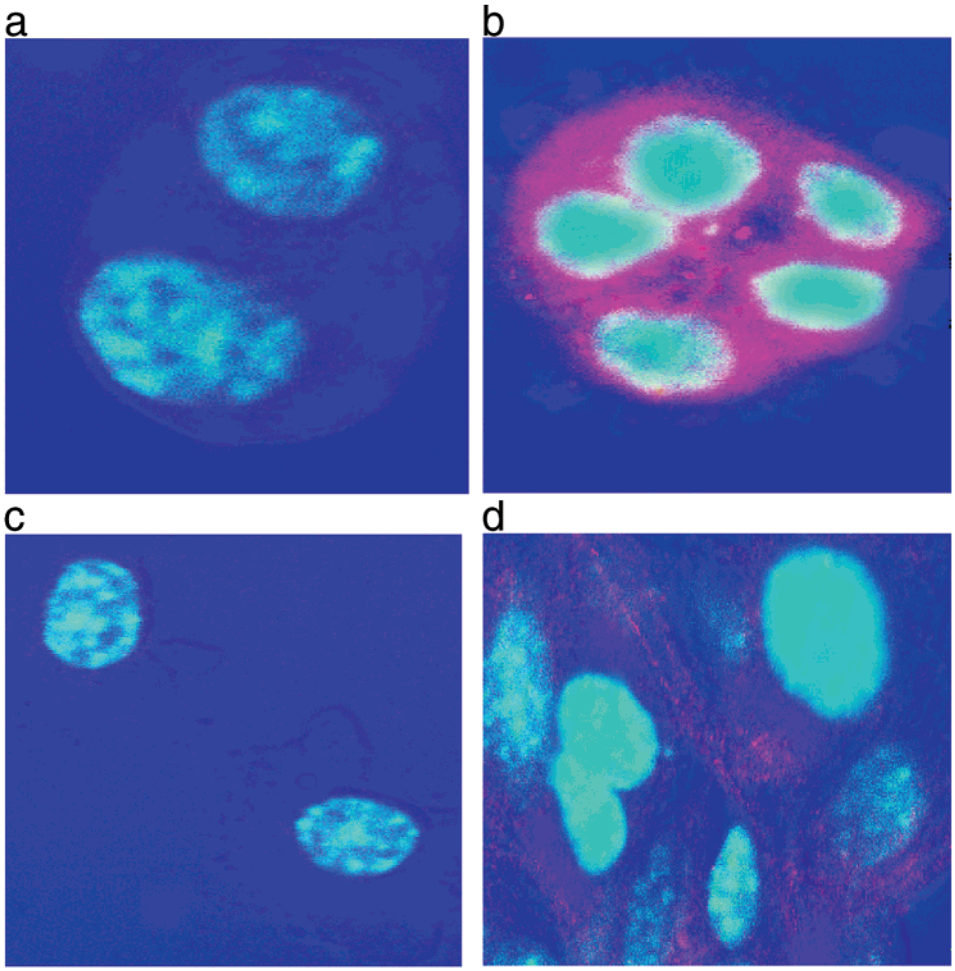


Figure 5. Confocal laser scanning micrographs of OV-1063 cells and C-26 cells treated with IC_{50} values of *trans*-[$PtCl_2$(4-pic)(pip)] for 5 h. Untreated (control) OV-1063 (a) and C-26 (c) and treated (b and d, respectively) cells growing on a glass coverslip were placed on a glass slide and viewed and photographed using a CLSM.

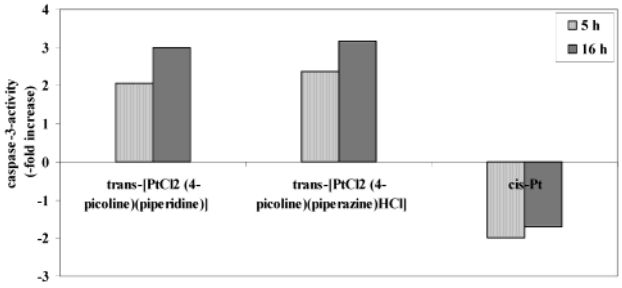


Figure 6. Caspase-3 activity in OV-1063 cells, which were treated for 5 or 16 h with IC_{50} values of *trans*-[$PtCl_2$(4-pic)(pip)] (6.5 μM), *trans*-[$PtCl_2$(4-pic)(pz)·HCl] (7.5 or 6.5 μM, respectively), or cisplatin (2 or 1 μM, respectively) as compared to untreated (control) cells. Both drug-treated and control cells were then harvested, lysed, and assayed after the indicated amount of time, as described in the kit protocol.

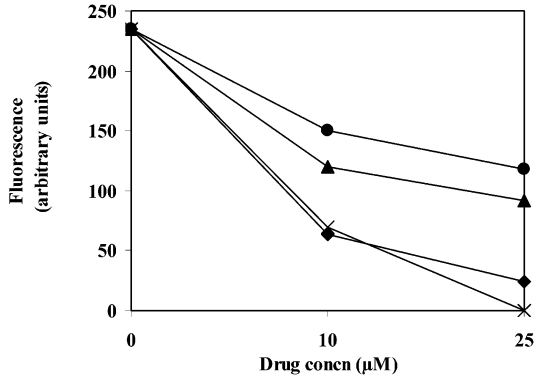


Figure 7. Dependence of EtBr fluorescence on concentration for DNA modified by various platinum complexes: cisplatin (filled diamonds), transplatin (filled circles), *trans*-[$PtCl_2$(4-pic)(pip)] (filled triangles), or *trans*-[$PtCl_2$(4-pic)(pz)·HCl] (×) in 10 mM $NaClO_4$ at 37 °C for 24 h. Measurements of EtBr fluorescence were performed on an LS50B luminescence spectrometer. Data points measured in triplicate varied on average by ±3%.

as $Pt(NH_3)_3Cl]Cl$ or transplatin are not cytotoxic to cancer cells even though they form adducts with cellular DNA. Some of the reasons for the lack of cytotoxicity can be attributed to differential repair processes or to the ability of DNA polymerases to perform trans lesion synthesis. Different types of lesions can have different effects on cellular events, and thus, no direct correlation between the number of the adducts and the cytotoxicity should be expected. Because the compounds tested induced apoptosis, it may be that the platination levels of the DNA are not the most important factor. We are currently studying the DNA adducts of the platinum complexes of pip and pz in order to examine their nature, assess their ability to block replication and transcription, whether they are recognized by HMG1 domain proteins, and whether the adducts are repaired by NER. Only after obtaining those results will a clearer picture emerge.

In terms of the SAR, replacing one or both amine ligands of transplatin with 4-pic, pip, or pz markedly increases the activity relative to transplatin. In these cell lines, replacing one amine by 4-pic and the other by pip or pz yielded compounds that were more active than the corresponding *trans*-[$PtCl_2(NH_3)$(pip/pz)] compounds (**II** and **III**).

Substituting one amine ligand of cisplatin with either 4-pic or with pip resulted in reduction of the cytotoxic activity relative to cisplatin. The large difference in activity between complexes **V** and **VI** is probably due to the differences in steric bulk, geometry, and flexibility of the ligands. The cytotoxic activity of the pip complex is very similar to that of cisplatin and is reminiscent of the properties of AMD473, which was designed to have reduced reactivity toward biological thiols and thioethers (proteins and peptides). This is considered beneficial since reaction of cisplatin with biological sulfur-containing nucleophiles is believed to be the source of acquired resistance and of the toxic side effects of the drug.

Similar cytotoxicity results were obtained when compounds **I**−**IV** were screened against the six human ovarian cell lines A2780, A2780cisR, CH1, CH1cisR, 41M, and 41McisR. All of these complexes were much more active than transplatin. Comparison of the cytotoxic profiles of the charged pz complexes with those of their neutral pip analogues showed that the charged complexes were superior in circumventing cisplatin resistance.[38]

Because apoptotic cells are phagocytized and are processed by macrophages, while necrotic cells release their constituents to the extracellular matrix, producing inflammation,[39] it may be advantageous to design platinum antitumor drugs that will selectively kill tumor cells by mean of apoptosis, avoiding the side effects of necrosis.

In summary, we have prepared novel *trans*-platinum compounds with significant cytotoxic activity against two lines of cancer cells. A positively charged complex, where the charge resides on the ligand, shows remarkable cell uptake and DNA binding. Two of these compounds induce apoptosis in ovarian OV-1063 cancer cell lines.

Experimental Section

Materials. Potassium tetrachloroplatinate (K_2PtCl_4), pip, pz, 4-methylpyridine, *tert*-butyl 1-pz carboxylate, cisplatin, and transplatin were purchased from Aldrich and were used without further purification.

Instrumentation. All NMR spectra were recorded on a Varian Inova 500 MHz spectrometer using a 5 mm switchable probe. ^{195}Pt NMR spectra were referenced externally to K_2PtCl_4 in HCl (−1624 ppm). Atomic absorption spectra were recorded on a flameless Zeeman atomic absorption spectrometer (FAAS).

Synthesis and Characterization of the Platinum Complexes. *trans*-[$PtCl_2$(4-pic)(pip)] (I). K_2PtCl_4 (200 mg, 0.482 mmol) was dissolved in 30 mL of doubly distilled water (DDW). A 2.5 equiv amount of 4-pic (117.3 μL, 1.2 mmol) was added, and the mixture was stirred overnight at room temperature. The yellow precipitate, *cis*-[$PtCl_2$(4-pic)$_2$] [^{195}Pt NMR(DMF) = −1964 ppm], was collected by filtration and washed with 50 mL of DDW and 40 mL of diethyl ether. *cis*-[$PtCl_2$(4-pic)$_2$] (226 mg, 0.5 mmol) was suspended in 40 mL of DDW with 2 equiv of pip (99 μL, 1 mmol), and the suspension was heated to 80 °C for 3 h. The solution turned clear and colorless with some formation of a black precipitate. The reaction mixture was allowed to cool to room temperature, and the precipitated material was filtered off. To the colorless filtrate, 4 mL of concentrated HCl was added and the mixture was heated to 90 °C. The heating was maintained for 6 h during which a yellow precipitate was formed. The reaction mixture was allowed to cool to room temperature, and the precipitate (180 mg) was collected and washed with 50 mL of DDW, 10 mL of EtOH, and 30 mL of diethyl ether. Yield: 81%. Anal. ($C_{11}H_{18}Cl_2N_2Pt$) C, H, N. ^{195}Pt NMR (DMF): −2087 ppm.

***trans*-[$PtCl_2(NH_3)$(4-pic)] (II).** *cis*-Diamminedichloroplatinum(II) (300 mg, 1 mmol) was suspended in 30 mL of DDW. Two equivalents of 4-pic (194.6 μL, 2 mmol) was added, and the suspension was heated to 85 °C for 3 h. During this time, the yellow suspension turned to a colorless clear solution (in some cases, a black precipitate formed). The reaction mixture was cooled to room temperature and filtered, and 3 mL of concentrated HCl was added dropwise. The temperature was elevated to 90 °C for 6 h during which the yellow product, *trans*-[$PtCl_2(NH_3)$(4-pic)] (**II**), precipitated. The reaction mixture was allowed to stand at room temperature for 4 h. The yellow product (550 mg) was collected by filtration and washed with 30 mL of DDW, 20 mL of EtOH, and 30 mL of diethyl ether. Yield: 73.1%. Anal. ($C_6H_{10}Cl_2N_2Pt$) C, H, N. ^{195}Pt NMR (DMF): −2021 ppm.

***trans*-[$PtCl_2(NH_3)$(pip)] (III).** *cis*-Diamminedichloroplatinum(II) (300 mg, 1 mmol) was suspended in 30 mL of DDW. Two equivalents of pip (198 μL, l mmol) was added, and the suspension was heated to 85 °C for 3 h. During this time, the yellow suspension turned to a colorless clear solution (in some cases, a black precipitate formed). The reaction mixture was cooled to room temperature, filtered, and 3 mL of concentrated HCl was added dropwise. The temperature was elevated to 90 °C for 6 h during which the yellow product, *trans*-[$PtCl_2(NH_3)$(pip)] (**III**), precipitated. The reaction mixture was allowed to stand at room temperature for 4 h. The yellow product (320 mg) was collected by filtration and washed with 40 mL of DDW, 10 mL of EtOH, and 40 mL of diethyl ether. Yield: 86.9%. Anal. ($C_5H_{14}Cl_2N_2Pt$) C, H, N. ^{195}Pt NMR (DMF): −2167 ppm.

***trans*-[$PtCl_2$(4-pic)(pz)]·HCl (IV).** The synthesis of this compound is described in ref 38.

***cis*-[$PtCl_2(NH_3)$(4-pic)] (V).** The synthesis of this complex is identical to that described for compound **VI** below except that 4-pic was used instead of pip. Overall yield for **V**: 63%. Anal. ($C_6H_{10}Cl_2N_2Pt$) C, H, N. ^{195}Pt NMR (DMF): −2021 ppm.

***cis*-[$PtCl_2(NH_3)$(pip)] (VI).** K_2PtCl_4 (415 mg,1 mmol) was dissolved in 50 mL of DDW, and 8 equiv of KI (1.328 g, 8 mmol) was added. The mixture was stirred at room temperature for 15 min, and then, 2 equiv of pip (198 μL, 2 mmol) was added slowly. The mixture was stirred for 1 h at room temperature, during which a yellow precipitate was formed. The precipitate was collected and washed thoroughly with 50 mL of DDW and with 20 mL of a (1:1) acetone:diethyl ether mixture. After it was dried, the yellow precipitate (500 mg, 0.8 mmol) was suspended in a mixture of 20 mL of DDW and 40 mL of ethanol, to which 1 mL of perchloric acid (70%) was added. The suspension was stirred at room temperature for 8 days. During this period, the yellow precipitate turned to brown. The brown precipitate was collected by filtration and washed with 40 mL of DDW and 20 mL of acetone:diethyl ether (1:1). After it was dried, the precipitate was resuspended in 20 mL of DDW, 0.5 mL of 25% NH_4OH was added dropwise, and the mixture was vigorously stirred for 24 h, during which the brown-colored precipitate turned to yellow. The yellow precipitate was collected and washed thoroughly with 50 mL of DDW and 10 mL of acetone:diethyl ether and dried by continuous suction. The product was characterized as the mixed *cis*-aminepiperidinediiodoplatinum(II). ^{195}Pt NMR (DMF): − 3260 ppm.

cis-Amminepiperidinediiodoplatinum(II) (300 mg, 0.54 mmol) was suspended in 20 mL of DDW, and 2 equiv of $AgNO_3$ (184.9 mg, 1.08 mmol) was added. The suspension was vigorously stirred in the dark for 24 h. The AgI precipitate was filtered

off, and the aqueous filtrate was transferred into a 50 mL vessel, to which 0.5 g of KCl was added. The colorless solution turned yellowish, and the dichlorodiamineplatinum(II) product started precipitating. After 4 h at room temperature, the yellowish precipitate (260 mg) was collected and washed thoroughly with 50 mL of DDW and dried by washing with 100 mL of diethyl ether. Overall yield of complex **VI**: 69%. Anal. ($C_5H_{14}Cl_2N_2Pt$) C, H, N. ^{195}Pt NMR (DMF): −2159 ppm.

Cell Cultures. Two tumor cell lines in monolayers were used. A human ovarian carcinoma cell line (OV-1063), established at the Hadassah University Hospital, and a human colon carcinoma cell line (C-26) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), antibiotics, and glutamine. All culture medium components were purchased from Biological Industries (Beit-HaEmek, Israel). Both cell lines were maintained at 37 °C in a water-jacketed CO_2 incubator.

MB Assay of Cell Survival. The cytotoxicity of these compounds was tested by the MB staining assay.[22] A known number of exponentially growing cells in 200 μL of medium were plated in 96 microwell, flat-bottomed plates. For each of the variants tested, four wells were used. Following 24 h of incubation in culture, 20 μL of different concentrations of the drugs was added to each well containing untreated cells. Normal saline was added to the controls. Cells were exposed to drugs for 4, 24, or 72 h. At the end of drug exposure, for a fixed time interval, the drug-treated cells, as well as parallel control cells, were washed, and the incubation was continued in fresh medium until termination of the experiment. Following 72 h of growth, cells were fixed by adding 50 μL of 2.5% glutaraldehyde to each well for 15 min. Fixed cells were rinsed 10 times with deionized water and once with borate buffer (0.1 M, pH 8.5), dried, and stained with MB (100 μL of 1% solution in 0.1 M borate buffer, pH 8.5) for 1 h at room temperature. Stained cells were rinsed thoroughly with deionized water to remove any noncell-bound dye and then dried. The MB bound to the fixed cells was extracted by incubation at 37 °C with 200 μL of 0.1 N HCl for 1 h, and the net optical density of the dye in each well was determined by a plate spectrophotometer (Labsystems Multyskan Bichromatic, Finland) at 620 nm.

The advantage of the MB method with 96 microwell plates is the possibility of running a wide range of experiments on the rate of cell proliferation and survival with a large number of data points, where cells are grown in the same plate and assayed exactly in the same conditions for different experimental variants. The validity of the MB assay for evaluating cell survival is supported by the high correlation between the MB colorimetric assay and colony-forming units assay results.[23]

Platinum Drug Intracellular Accumulation Measurement. Cells were seeded for 48 h before one of the drugs was added to the culture medium. After 24 h of exposure, drugs were removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and pelleted. Cells (1×10^6) were dried and mineralized by heating for 10 min in 65% HNO_3 (BDH, England).[24] Samples were dissolved in deionized water, and each sample was measured at two different dilutions by FAAS. The calibration curves included five standards of K_2PtCl_4 stock solution with concentrations ranging from 50 to 250 ng of platinum per mL. Platinum content was expressed as picomoles platinum per 1×10^6 cells.

Determination of Pt−DNA Adducts by FAAS. Cells were seeded for 48 h before one of the drugs was added to the culture medium. After 24 h of exposure, the drugs were removed and the cells were washed twice with ice-cold PBS and pelleted. DNA from platinum-containing material (2×10^6 cells) was extracted from the cell pellet with the QIAamp DNA Blood Kit (QIAGEN, Germany) according to the manufacturer's instructions. DNA yield was determined by measuring the absorbance at 260 nm of the eluate. The DNA isolated from each sample averaged 50 ± 10 μg/mL. Purity was determined by calculating the ratio of absorbance at 260−280 nm; the grade of purification of DNA was on the average 95%.

Assessment of Apoptosis. Apoptosis was assessed by staining of the C-26 and OV-1063 cells with Merocyanine 540 (MC 540) and 4′,6-diamidino-2-phenylindone dihydrochloride (DAPI), both from Molecular Probe, Eugene, OR. This assay is based on the observation that soon after the initiation of apoptosis, PS translocates from the inner face of the plasma membrane to the cell surface. At this point, PS can be detected readily by staining with MC 540, which has a strong affinity to PS.[25] Chromatin condensation was assessed by staining with DAPI, which preferentially stains double-stranded DNA.

In our experiments, samples containing 5×10^5 cells were cultured on six well plates covered with a glass coverslip. After treatment of the cells with IC_{50} concentrations of the drugs, cells were washed with PBS and incubated for 2 min in the dark in 500 μL of PBS containing 2.5 μL of MC 540 (1 mg/mL). Subsequently, cells were washed with PBS, fixated with 4% formaldehyde, and stained with 300 μL of DAPI (3 μM). Thereafter, a glass coverslip was placed on a glass slide, which was then photographed using a confocal laser scanning microscope (CLMS) (Zeiss 410, Germany), a high-resolution microscope that allows viewing and quantification of fluorescence in different cell compartments.

Biochemically, apoptosis was checked by the EnzChek Caspase-3 Assay Kit (Molecular Probes). This allows the detection of apoptosis by assaying for increases in caspase-3 and other DEVD-specific protease activities (e.g., caspase-7). The basis for the assay is rhodamine 110 bis-(*N*-CBZ-aspartyl-L-glutamyl-L-valyl-aspartic acid amide), Z-DEVD-R110. This substrate is a bisamide derivative of rhodamine 110 (R110) containing DEVD peptides covalently linked to each of R110's amino groups. Upon enzymatic cleavage, the nonfluorescent bisamide substrate was converted to the fluorescent R110, which was quantified by a fluorescence microplate reader using excitation at 485 ± 10 nm and detection at 535 ± 10 nm. Briefly, C-26 and OV-1063 cells were treated with IC_{50} of *trans*-[$PtCl_2$(4-pic)(pip)] (4.5 and 6.5 μM, respectively) and of *trans*-[$PtCl_2$(4-pic)(pz)]HCl (5 and 7.5 μM, respectively) for 5 or 16 h. Both induced and control cells were then harvested and lysed. Enzyme reactions were performed in 96 well plates with 50 μg of cytosolic proteins (55 min of incubation) and a final concentration of 25 μM Z-DEVD-R110 substrate, as described in the kit protocol.

Determination of Pt−DNA Adducts byEtBr Fluorescence. The plasmid pS16-hGH, a 4.8 kbp plasmid containing a gene coding for human growth hormone, was prepared as previously described.[26] The freshly prepared DNA was analyzed by agarose gel (1%) electrophoresis using poststaining with SYBR Grease I fluorescent dye (Molecular Probes). Quantitative analysis of supercoiled plasmid[27] was performed and showed that the plasmid DNA was 85−90% in a supercoiled form. UV-spectroscopy showed no presence of protein or RNA contamination in any of the DNA batches: the ratio of absorbance at 260 nm to that at 280 nm was always between 1.8 and 1.9.

DNA was reacted with various platinum complexes in 10 mM $NaClO_4$ (pH 7.0) at 37 °C in the dark for 24 h.[14] Measurements of EtBr fluorescence were performed on an LS50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT). Fluorescence measurements of DNA modified by platinum in the presence of EtBr were performed using the excitation wavelength of 546 nm (slit 10 nm) and emission wavelength of 590 nm (slit 10 nm) at 25 °C. The concentrations were 10 μg/mL for DNA and 40 μg/mL for EtBr, which corresponded to the saturation of all intercalation sites of EtBr in DNA.[28]

Acknowledgment. Y.N. thanks the David R. Bloom Center for Pharmacy at The Hebrew University of Jerusalem, Israel, for financial support. D.G. thanks the Israel Cancer Association (Grant 20010031-B) for support of this project. This study was partially supported by the Belfer foundation and the Barenholz Fund.

References

(1) O'Dwyer, P. J.; Steveson, J. P.; Johnson, S. W. Clinical Status of Cisplatin, carboplatin and other platinum based antitumor drugs. In *Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug*; Lippert, B., Ed.; Verlag Helvetica Chimica Acta: Zurich, 1999; pp 31−71.

(2) Weiss, R. B.; Christian, M. C. New cisplatin analogues in development. *Drugs* **1993**, *46*, 360.

(3) Jamieson, E. R.; Lippard, S. J. Structure, recognition, and processing of Cisplatin-DNA Adducts. *Chem. Rev.* **1999**, *99*, 2467−2498.

(4) Hickman, J. A. Apoptosis induced by anticancer drugs. *Cancer Metastasis Rev.* **1992**, *11*, 121−139.

(5) Kartalou, M.; Essigmann, J. M. Recognition of cisplatin adducts by cellular proteins. *Mutat. Res.* **2001**, *478*, 1−21.

(6) Wong, W.; Giandomenico, C. M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **1999**, *99*, 2451−2466.

(7) Judson, I.; Kelland, L. R. New developments and approaches in the platinum arena. *Drugs* **2000**, *59*, 29−36.

(8) Cleare, M. J.; Hoeschele, J. D. Studies on the antitumor activity of group VIII transition metal complexes. Part I. Platinum(II) complexes. *Bioinorg. Chem.* **1973**, *2*, 187−210.

(9) Hollis, L. S.; Amundsen, A. R.; Stern, E. W. Chemical and biological properties of a new series of *cis*-diamineplatinum(II) antitumor agents containing three nitrogen donors: *cis*-[Pt-$(NH_3)_2$(N-donor)Cl]$^+$. *J. Med. Chem.* **1989**, *32*, 128−136.

(10) Hollis, L. S.; Sundquist, W. I.; Burstyn, J. N.; Heiger-Bernays, W. J.; Bellon, S. F.; Ahmed, K. J.; Amundsen, A. R.; Stern, E. W.; Lippard, S. J. Mechanistic studies of a novel class of trisubstituted platinum(II) antitumor agents. *Cancer Res.* **1991**, *51*, 1866−1875.

(11) Farrell, N.; Ha, T. T.; Souchard, J. P.; Wimmer, F. L.; Cross, S.; Johnson, N. P. Cytostatic trans-platinum(II) complexes. *J. Med. Chem.* **1989**, *32*, 2240−2241.

(12) Farrell, N.; Kelland, L. R.; Roberts, J. D.; Van Beusichem, M. Activation of the trans geometry in platinum antitumor complexes: a survey of the cytotoxicity of trans complexes containing planar ligands in murine L1210 and human tumor panels and studies on their mechanism of action. *Cancer Res.* **1992**, *15*, 5065−5072.

(13) Montero, E. I.; Diaz, S.; Gonzalez-Vadillo, A. M.; Perez, J. M.; Alonso, C.; Navarro-Ranniger, C. Preparation and characterization of novel *trans*-[$PtCl_2$(amine)(isopropylamine)] compounds: cytotoxic activity and apoptosis induction in ras-transformed cells. *J. Med. Chem.* **1999**, *42*, 4264−4268.

(14) Perez, J. M.; Montero, E. I.; Gonzalez, A. M.; Alvarez-Valdez, A.; Alonso, C.; Navarro-Ranniger, C. Apoptosis induction and inhibition of H-ras overexpression by novel *trans*-[$PtCl_2$-(isopropylamine)(amine)] complexes. *J. Inorg. Biochem.* **1999**, *77*, 37−42.

(15) Coluccia, M.; Nassi, A.; Boccarelli, A.; Giordano, D.; Cardellicchio, N.; Intini, F. P.; Natile, G.; Barletta, A.; Paradiso, A. In vitro antitumour activity and cellular pharmacological properties of the platinum-iminoether complex *trans*-[$PtCl_2$[E-HN═C(OMe)-Me]$_2$]. *Int. J. Oncol.* **1999**, *15*, 1039−1044.

(16) Qu, Y.; Rauter, H.; Fontes, A. P. S.; Bandarage, R.; Kelland, L. R.; Farrell, N. Synthesis, characterization, and cytotoxicity of trifunctional dinuclear platinum complexes: comparison of effects of geometry and polyfunctionality on biological activity. *J. Med. Chem.* **2000**, *43*, 3189−3192.

(17) Perez, J. M.; Fuertes, M. A.; Alonso, C.; Navarro-Ranninger, C. Current status of the development of *trans*-platinum antitumor drugs. *Crit. Rev. Oncol. Hematol.* **2000**, *35*, 109−120.

(18) Natile, G.; Coluccia, M. Current status of *trans*-platinum compounds in cancer therapy. *Coord. Chem. Rev.* **2001**, *216*, 383−410.

(19) (a) Low, S. W.; Ruley, E. H.; Jacs, T.; Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **1993**, *74*, 957−967. (b) Jones, N. A.; Turner, J.; McIlwarts, A. J.; Brown, R.; Dive, C. Cisplatin and paclitaxel induced apoptosis of ovarian carcinoma cells and the relationship between bax and bak up-regulation and the functional status of p53. *Mol. Pharmacol.* **1998**, *53*, 819−826.

(20) Perez, J. M.; Montero, E. I.; Gonzalez, A. M.; Alvarez-Valdes, A.; Alonso, C.; Navarro-Ranninger, C. J. Apoptosis induction and inhibition of H-ras overexpression by novel *trans*-[$PtCl_2$-(isopropylamine)(amine)] complexes. *Inorg. Biochem.* **1999**, *77*, 37−42.

(21) Perez, J. M.; Camazon, M.; Alvarez-Valdes, A.; Quiroga, A. G.; Kelland, L. R.; Alonso, C.; Navarro-Ranninger, C. Synthesis, characterization and DNA modification induced by a novel Pt-(IV)-bis(monoglutarate) complex which induces apoptosis in glioma cells. *Chem. Biol. Interact.* **1999**, *117*, 99−115.

(22) Gorodetsky, R.; Levy-Acaba, F.; Mou, X.; Vexler, A. M. Combination of cisplatin and radiation in cell culture: effect of duration of exposure to drug and timing of irradiation. *Int. J. Cancer* **1998**, *75*, 635−642.

(23) Gorodetsky, R.; Moy, X.; Pfeffer, M. R.; Peretz, T.; Levy-Agababa, F.; Vexler, A. M. Sub-additive effect of the combination of radiation and cisplatin in cultured murine and human cell lines. *Isr. J. Med. Sci.* **1995**, *31*, 175−180.

(24) Lindauer, E.; Holler, E. Cellular distribution and cellular reactivity of platinum(II) complexes**.** *Biochem. Pharmacol.* **1996**, *52*, 7−14.

(25) Reid, S.; Cross, R.; Snow, E. C. Combined Hoechst 33342 and merocyanine 540 staining examine murine B cell cycle stage, viability and apoptosis. *J. Immunol. Methods* **1996**, *192*, 43−54.

(26) Zuidan, N. J.; Hirsh-Lerner, D.; Margulies, S.; Barenholz, Y. Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency**.** *Biochim. Biophys. Acta* **1999**, *1419*, 207−220.

(27) Even-Chen, S.; Barencholz, Y. DOTAP cationic liposomes prefer relaxed over supercoiled plasmide. *Biochim. Biophys. Acta* **2000**, *1509*, 176−188.

(28) Butour, J. L.; Macquet, J. P. Differentiation of DNA-platinum complexes by fluorescence. The use of an intercalating dye as a probe. *Eur. J. Biochem.* **1977**, *20*, 455A−463A.

(29) (a) Ciccarese, A.; Clemente, D. A.; Fanizzi, F. P.; Marzotto, A.; Valle, G. Boat versus chair conformation in *N*-methyl- and *N*,*N*′-dimethylpiperazine platinum(II) complexes studied by X-ray analysis. A rare example of metal chelate piperazine: *cis*-[$PtCl_2$(Me_2ppz)]. Part I. *Inorg. Chim. Acta* **1998**, *275*−*276*, 410−418. (b) Ciccarese, A.; Clemente, D. A.; Fanizzi, F. P.; Marzotto, A.; Valle, G. Synthesis and molecular structure of *N*-methylpiperazinium(+1) and *N*,*N*′-dimethylpiperazinium(+2) platinum-(II) and platinum(IV) complexes. Part II. *Inorg. Chim. Acta* **1998**, *275*−*276*, 419−426.

(30) Steller, H. Mechanisms and genes of cellular suicide. *Science* **1995**, *267*, 1445−1449.

(31) Martin, S. J.; Reutelingsperger, C. P. M.; Rader, A. J.; van Schle, R. C. A. A.; LaFace, D. M.; Green, D. R. Early redistribution of plasma membrane phosphatidylserine is a general future of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and AbI. *J. Exp. Med.* **1995**, *182*, 1545−1556.

(32) Thornberry, N. A.; Lazebnic, Y. Caspases: enemies within. *Science* **1998**, *281*, 1312−1316.

(33) Villa, P.; Kaufmann, S. H.; Earnshaw, W. C. Caspases and caspase inhibitors. *Trends Biochem. Sci.* **1997**, *22*, 388−393.

(34) Ciesielska, E.; Studzan, K.; Zyner, E.; Ochocki, J.; Szmigiero, L. DNA damage and apoptosis induction in L1210 cells by diamminedichloroplatinum(II) and its new aminoflavone analogue, *Cell. Mol. Biol. Lett.* **2000**, *5*, 441−450.

(35) Liu, W.; Davis, D. W.; Ramirez, K.; McConkey, D. J.; Ellis, L. M. Endothelial cell apoptosis is inhibited by a soluble factor secreted by human colon cancer cells. *Int. J. Cancer* **2001**, *92*, 26−30.

(36) Bellone, G.; Garbone, A.; Sibona, N.; Bosco, O.; Tibaudi, D.; Smirne, C.; Martone, T.; Gramigni, C.; Camandona, M.; Emanuelli, G.; Rodeck, U. Aberrant activation of c-kit protects colon carcinoma cells against apoptosis and enhances their invasive potential. *Cancer Res.* **2001**, *61*, 2200−2206.

(37) Perego, P. Caserini, C.; Gatti, L.; Carenini, N.; Romanelli, S.; Supino, R.; Colangelo, D.; Viano, I.; Leone, R.; Spinelli, S.; Pezzoni, G.; Manzotti, C.; Farrell, N.; Zunino, F. A novel trinuclear platinum complex overcomes cisplatin resistance in an osteosarcoma cell system. *Mol. Pharmacol.* **1999**, *55*, 528−534.

(38) Najajreh, Y.; Perez, J. M.; Navarro-Ranninger, C.; Gibson, D. *J. Med. Chem.* **2002**, *45*, 5189−5195.

(39) Wyllie, A. H.; Kerr, J. F. R.; Curie, A. R. Cell death: the significance of apoptosis. In *International Review of Cytology*; Bourne, G. H., Danielli, F. J., Jeon, K. W., Eds.; Academic Press: New York, 1980; pp 68, 251−306.

JM020817Y