Cationic Nonsymmetric Transplatinum Complexes with Piperidinopiperidine Ligands. Preparation, Characterization, in Vitro Cytotoxicity, in Vivo Toxicity, and Anticancer Efficacy Studies

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A series of complexes of the general formula *trans*-[$PtCl_2$(Am)(pip-pip)]·HCl where pip-pip is 4-piperidinopiperidine and Am is NH_3, methylamine (MA), dimethylamine (DMA), *n*-propylamine (NPA), isopropylamine (IPA), *n*-butylamine (NBA), or cyclohexylamine (CHA) were prepared and characterized, and their cytotoxic properties against ovarian and colon cancer cells were evaluated. The *trans*-[$PtCl_2(NH_3)$-(pip-pip)]·HCl was significantly more potent than cisplatin in all the cisplatin-resistant ovarian cancer cell lines and was nearly as cytotoxic as cisplatin against colon cancer cells. In vivo studies in mice showed that the pip-pip complexes are significantly less toxic than cisplatin. Cisplatin was more efficacious than both *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl in the A2780 and A2780cisR tumor xenograft models, consistent with its lower IC_{50} values in A2780 cells but contrary to the higher IC_{50} values in A2780cisR cells. In the colon cancer cell studies, *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl was slightly less potent than cisplatin in the in vitro studies but had efficacy comparable to that of cisplatin in the in vivo xenograft model.

Introduction

Since the discovery of the anticancer activity of cisplatin (Figure 1a) in 1966 and its subsequent approval by the FDA[a] as an anticancer drug, intensive efforts have been channeled toward the discovery of additional platinum anticancer agents.[1,2] Only two more platinum complexes have received FDA approval as anticancer drugs: carboplatin and oxaliplatin (parts b and c of Figure 1, respectively).[3] All three drugs are neutral and bifunctional, have the two am(m)ine ligands in the cis orientation, and can be considered analogues of cisplatin. For many years it was assumed that platinum complexes having the trans geometry are incapable of cytotoxic activity.[4] In the 15 years that elapsed since Farrell first reported on the activation of the trans geometry for platinum complexes, it has become evident that not only can *trans*-diaminedichloroplatinum(II) complexes possess cytotoxic activity, but often they are superior to their cis analogues, particularly in cisplatin-resistant cell lines.[5−7] The so-called "nonclassical platinum complexes" such as *trans*-diaminedichloroplatinum(II) complexes with planar amine ligands (see Figure 1d for an example), bulky aliphatic amines (Figure 1e), iminoether (Figure 1f), piperidine (Figure 1g), and piperazine (Figure 1f) ligands are reported to have significant cytotoxicity.[8−12]

Not only did the early publications suggest that cytotoxic platinum complexes must have the cis geometry but they also stated that electroneutrality was important for activity. Hollis was the first to demonstrate that cationic complexes can be cytotoxic, and over time other cytotoxic charged platinum complexes were reported.[13−17] We have shown that platinum-(II) complexes with the nonplanar heterocyclic piperazine ligands, in which the positive charge is removed from the metal center, are quite effective against cisplatin-resistant human ovarian cancer cell lines. We prepared cationic piperazine complexes expecting that the solubility and hence the bioavailability of the charged platinum complexes would be significantly higher than those of their neutral counterparts.[10] Their cellular accumulation and their cellular DNA binding levels are significantly higher than their neutral piperidine counterparts.[10]

We have recently shown that the complex *trans*-[$PtCl_2(NH_3)$-(pip)] seems to act by a mechanism that is different from that of cisplatin.[18] Unlike cisplatin, carboplatin, and oxaliplatin, it does not form 1,2-intrastrand cross-links with DNA but forms mainly stable 1,3-intrastrand cross-links with ds-DNA. The 1,3-intrastrand cross-link is not recognized by HMG1 proteins, is not efficiently repaired by NER, and is more effective than cisplatin in inhibiting DNA replication. These results, combined with the recently reported results on the cytotoxicity of the charged *trans*-[$PtCl_2(NH_3)$(pz)]$^+$, prompted us to design, prepare, and evaluate the cytotoxic and DNA binding properties of a series of *trans*-[$PtCl_2$(Am)(pip-pip)]$^+$ complexes where pip-

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[a] Abbreviations: FDA, Food and Drug Administration; DDW, doubly distilled water; SAR, structure−activity relationship; pip-pip, 4-piperidinepiperidine; pz, piperazine; pip, piperidine; DMF, dimethylformamide; ESIMS, electrospray ionization mass spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; transplatin, *trans*-diamminedichloroplatinum(II); MA, methylamine; DMA, dimethylamine; NBA, *n*-butylamine; IPA, isopropylamine; CHA, cyclohexylamine; HMG1, high-mobility group; NER, nuclear excision repair; TXRF, total X-ray fluorescence; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); MB methylene blue; DMEM, Dulbecco's modified Eagle's medium.

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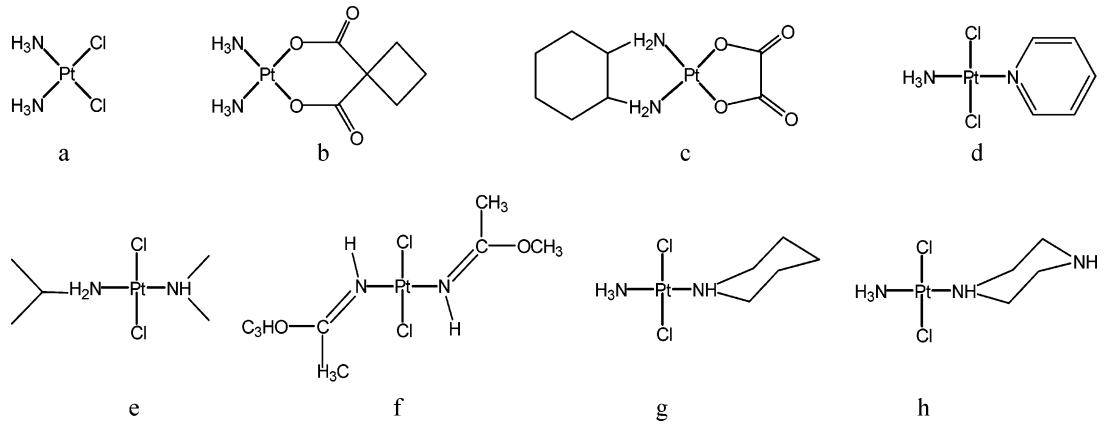


Figure 1. Cytotoxic platinum(II) compounds: (a) cisplatin; (b) carboplatin; (c) oxaliplatin; (d) *trans*-[$PtCl_2(NH_3)$(pyridine)]; (e) *trans*-[$PtCl_2$-(dimethylamine)(isopropylamine)]; (f) *trans*-[$PtCl_2$(iminoether)$_2$]; (g) *trans*-[$PtCl_2(NH_3)$(piperidine)]; (h) *trans*-[$PtCl_2(NH_3)$(piperazine)].

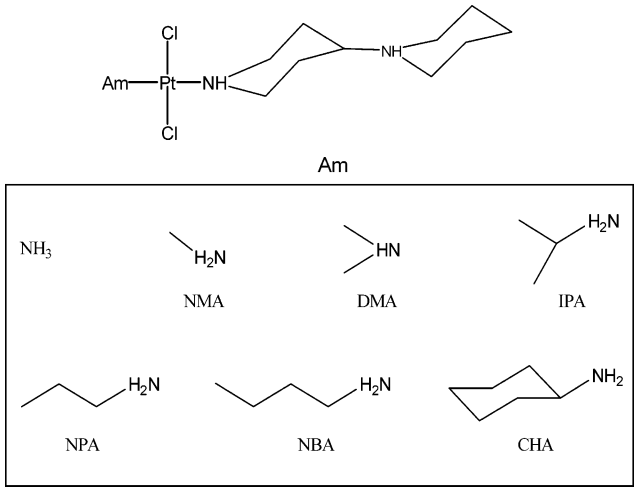


Figure 2. The *trans*-[$PtCl_2$(Am)(pip-pip)]·HCl complexes used in this study.

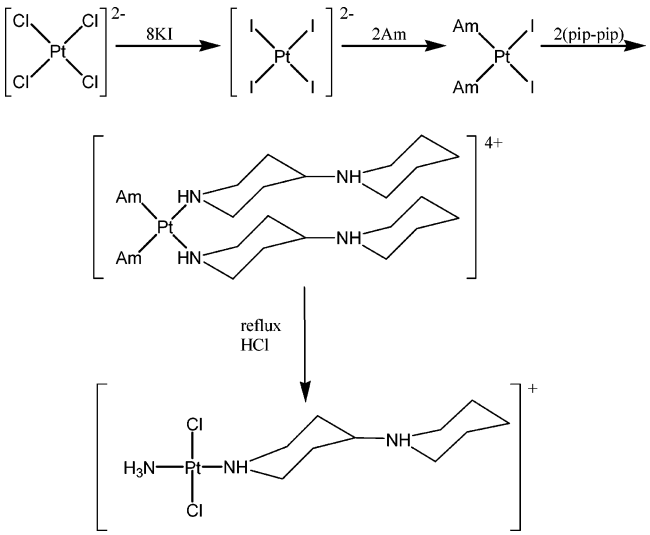
pip is 4-piperidinopiperidine and Am is NH_3, methylamine (MA), dimethylamine (DMA), *n*-propylamine (NPA), isopropylamine (IPA), *n*-butylamine (NBA), or cyclohexylamine (CHA) (Figure 2).

Results

1. Synthesis of the Platinum Complexes. Seven *trans*-dichlorodiam(m)ine platinum(II) complexes having the general formula *trans*-[$PtCl_2$(Am)(pip-pip)]·HCl where Am is NH_3, MA, DMA, IPA, NPA, NBA, or CHA were prepared. The syntheses were carried out essentially in the manner previously reported by us.[10,11] All compounds were characterized by ^{195}Pt NMR spectroscopy and by electrospray ionization mass spectrometry (ESIMS), and the purity of the compounds was ascertained by elemental analysis and by reversed-phase HPLC chromatography.

The procedure included the synthesis of the corresponding diam(m)inediiodo complexes, which were in turn transformed to the tetraam(m)ineplatinum(II) intermediates by replacing the iodide ligands with 2 equiv of pip-pip in DMF (Scheme 1). Because of steric hindrance, complete conversion to the tetraamine required warming the solutions to 60−70 °C for several hours. The conversion of the tetraamine to the corresponding *trans*-diam(m)inedichloroplatinum(II) was accomplished by reflux with aqueous hydrochloric acid. The duration of the reflux was dependent on the am(m)ine trans to the pip-pip (Scheme 1) and was determined by monitoring the progress of the reaction by ^{195}Pt NMR. The final complexes were purified as hydrochloride salts by recrystallization from aqueous hydrochloric acid solutions.

Scheme 1



2. Solubility of the Complexes. Generally, neutral diaminedicholroplatinum(II) complexes have limited solubility in aqueous media. This limits the bioavailability of platinum(II) complexes, especially for the trans isomers. To improve the solubility of trans complexes, a positive charge was added by using ligands that, in addition to the amine that coordinates to the Pt(II) center, have another amine (which is protonated at physiological pH) that is removed from the metal. The complexes are charged, but the metal center retains the neutral N_2Cl_2 coordination sphere. We demonstrated that transplatinum compounds with piperazine ligands are much more soluble than their neutral piperidine analogues.[10] Compounds of the general formula *trans*-[$PtCl_2$(Am)(pip-pip)]·HCl (**I**−**VII**) are significantly more soluble at neutral pH than neutral *trans*-diaminedichloroplatinum(II) complexes, having solubilities in the range 4.5−7.2 mM compared with 0.8 mM for transplatin.

3. Studies with Ovarian Cancer Cells. 3.1. Cytotoxicty Studies in Ovarian Cancer Cells. The cytotoxic activity of compounds **I**−**VII** was evaluated against three pairs of cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines (A2780/A2780cisR, 41M/41McisR, and CH1/CH1cisR).[19] These pairs of cell lines were selected on the basis of encompassing all of the known major mechanisms of resistance to cisplatin, 41McisR being resistant primarily through reduced drug transport, CH1cisR through enhanced DNA repair/tolerance, and

Table 1. IC_{50} Mean Values Obtained for Compounds **I**−**VII** after 24 h of Incubation

		$IC_{50} \pm SD^a$ (μM)					
	compd	A2780	A2780cisR	CH1	CH1cisR	41M	41McisR
I	*trans*-$PtCl_2(NH_3)$(pip-pip)	21 ± 2	24 ± 4 (1.1)	12 ± 3	15 ± 3 (1.3)	28 ± 3	48 ± 3 (1.7)
II	*trans*-$PtCl_2$ (MA)(pip-pip)	14 ± 1	29 ± 2 (2.0)	14 ± 2	38 ± 3 (2.7)	47 ± 5	124 ± 9 (2.6)
III	*trans*-$PtCl_2$ (DMA)(pip-pip)	24 ± 2	38 ± 3 (1.6)	36 ± 4	70 ± 6 (1.9)	47 ± 4	181 ± 15 (3.8)
IV	*trans*-$PtCl_2$(NPA)(pip-pip)	16 ± 2	30 ± 3 (1.9)	25 ± 2	39 ± 3 (1.6)	55 ± 4	115 ± 9 (2.1)
V	*trans*-$PtCl_2$(IPA)(pip-pip)	12 ± 1	33 ± 3 (2.8)	44 ± 3	57 ± 6 (1.3)	70 ± 6	147 ± 10 (2.1)
VI	*trans*-$PtCl_2$(NBA)(pip-pip)	14 ± 1	27 ± 2 (1.9)	35 ± 2	70 ± 5 (2.0)	67 ± 5	135 ± 8 (2.0)
VII	*trans*-$PtCl_2$(CHA)(pip-pip)	30 ± 3	45 ± 4 (1.5)	35 ± 2	72 ± 7 (2.1)	40 ± 3	194 ± 14 (4.8)
	trans-$PtCl_2(NH_3)$(pz)[b]	5 ± 1	44 ± 4 (8.8)	12 ± 3	34 ± 4 (2.8)	52 ± 5	155 ± 12 (2.9)
	trans-$PtCl_2$(pip)(pz)[b]	18 ± 2	64 ± 5 (3.6)	22 ± 3	85 ± 7 (3.9)	37 ± 4	118 ± 9 (3.2)
	trans-$PtCl_2$(NBA)(pz)[b]	16 ± 2	28 ± 2 (1.8)	17	18 ± 3 (1.1)	34 ± 5	49 ± 3 (1.4)
	trans-$PtCl_2(NH_3)_2$	>200	>200	>200	>200	>200	>200
	cis-$PtCl_2(NH_3)_2$	2.2 ± 0.6	38 ± 3 (17.3)	6 ± 1	23 ± 3 (3.8)	26 ± 2	107 ± 8 (4.1)

[a] The numbers in parentheses are the values for RF = (IC_{50} resistant cell line)/(IC_{50} sensitive cell line). [b] From ref 10.

A2780cisR through a combination of decreased uptake, enhanced DNA repair/tolerance, and elevated GSH levels.[20−22]

The IC_{50} values (compound concentration, in μM, that induces 50% cell death) of complexes **I**−**VII** were determined using the MTT method. The results appear in Table 1. Replacing one ammine in the inactive *trans*-$[PtCl_2(NH_3)_2]$ with a pip-pip ligand enhances the cytotoxicity relative to transplatin by a factor of 8−16 against the sensitive cell lines (>200 for TDDP vs 21 ± 2 for *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]^+$ (**I**) against A2780 cells; 12 ± 3 against CH1 cells, and 28 ± 3 against 41M cells). *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]^+$ (**I**) is as potent as cisplatin against sensitive 41M cells, nearly half as potent against CH1 cells, and 10-fold less potent against A2870. When the IC_{50} of this complex was evaluated against the cisplatin-resistant cell lines, complex **I** proved to be more potent than cisplatin against all three resistant lines (IC_{50}: 24 ± 4, 15 ± 3, 48 ± 3 μM for **I** vs 38 ± 3, 23 ± 3, 107 ± 8 μM for cisplatin, respectively). Also, the RF (resistance factor, defined as IC_{50}(resistant)/IC_{50}(sensitive)) values of **I** were less than those of cisplatin in all three pairs (RF(**I**) = 1.14, 1.25, 1.71 vs 17.27, 3.83, 4.12 for the A2780/A2780cisR, CH1/CH1cisR, 41M/41McisR cell lines, respectively).

Cytotoxicity studies of *trans*-$[PtCl_2(Am)(pip\text{-}pip)]$·HCl complexes **II**−**VII** against A2780 revealed that these trans complexes are cytotoxic with IC_{50} values in the 15−30 μM range, more than 7-fold more active than parent transplatin but less potent than cisplatin. Against the A2780cisR cell line, the IC_{50} values were around 30 μM (compared with 38 μM for cisplatin). The RF values were more than 6-fold smaller than that of cisplatin, indicating that the cellular resistance mechanisms are less effective against these compounds than against cisplatin.

It is interesting to compare compounds **I** and **II**, which differ only by a CH_2 group. *trans*-$[PtCl_2(CH_3NH_2)(pip\text{-}pip)]$·HCl (**II**), which is more hindered than *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]$·HCl **(I)**, is 50% more potent than **I** against the sensitive A2780 cell line (IC_{50} = 21 ± 2 μM for **I** vs 14 ± 1 μM for **II**). In the CH1 cells, compounds **I** and **II** have similar potencies, while in the 41M cells **I** is 50% more potent than **II**. Interestingly, in the resistant cell line A2780cisR, **I** is only slightly more potent than **II** (24 ± 4 and 29 ± 2 μM for **I** and **II**, respectively), but in CH1cisR and 41McisR cell lines, **I** is more than twice as potent as **II** (15 ± 3 and 48 ± 3 μM vs 38 ± 3 and 124 ± 9 μM for **I** and **II**, respectively).

Nearly all the disubstituted complexes were much more active against the resistant A2780cisR line than against the other two resistant lines CH1cisR and 41McisR.

3.2. Cellular Accumulation and Nuclear DNA Platination Studies. Cellular accumulation and nuclear DNA platination studies were carried out to address the concern that charged complexes may not be internalized by cells as efficiently as cisplatin and to check whether the large differences between the cytotoxicities of *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]$·HCl and *trans*-$[PtCl_2(CH_3NH_2)(pip\text{-}pip)]$·HCl stem from differential cellular accumulation or from significantly different levels of DNA platination. The cell accumulation experiments and the quantification of DNA platination were performed for compounds **I**, **II**, and cisplatin as previously described.[18] The results of the cellular accumulation and DNA platination of the three compounds in two resistant cell lines, A2780cisR and 41McisR, are depicted in Figure 3.

In the 41McisR cell line *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]$·HCl accumulates in the cells significantly more quickly than cisplatin, which is slightly faster than *trans*-$[PtCl_2(CH_3NH_2)(pip\text{-}pip)]$·HCl (Figure 3A). It is not clear why substituting the ammine ligand by a methylamine should significantly affect cell accumulation. The levels of cell-associated Pt mirror the rate of accumulation: *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]$·HCl > cisplatin > *trans*-$[PtCl_2(CH_3NH_2)(pip\text{-}pip)]$·HCl (Figure 3A). Both the cellular accumulation and DNA binding (Figure 3B) correlate directly with the IC_{50} values. In the case of A2780cisR, the order of accumulation was **I** ≫ **II** > cisplatin (Figure 3C) and the DNA platination levels of **I** and **II** were nearly the same and greater than that of cisplatin (Figure 3D). In A2780cisR cells, the IC_{50} values correlate with both cellular accumulation and DNA platination.

3.3. Effect of Dextrose and Saline on the Cytotoxicity. Since in vivo studies are often performed by administering the drug in a saline solution or in dextrose, we decided to carry out cytotoxicity studies by diluting the compounds in either dextrose or saline in order to select the best approach for in vivo studies. The results of these studies appear in Table 2.

The compounds were solubilized in 0.9% sodium chloride or at the same concentration in 5% dextrose. The most important observation was the potentiation of the activity by a factor of ∼2 against both sensitive and resistant cell lines when positively charged compounds were dissolved in 5% dextrose prior to in vitro testing. For *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]$·HCl the IC_{50} decreased from 9 ± 2 μM in 0.9% NaCl to 3.7 ± 0.58 μM in 5% dextrose in the sensitive A2780 cells. In the resistant A2780cisR cells, the IC_{50} decreased from 30 ± 3.5 μM in 0.9% NaCl to 12.5 ± 1 μM in 5% dextrose. Similar results were obtained with *trans*-$[PtCl_2(NBA)(pip\text{-}pip)]$·HCl.

3.4. In Vivo Studies in Nude Mice Bearing A2780 and A2780cisR Tumors. On the basis of the in vitro studies, *trans*-$[PtCl_2(NH_3)(pip\text{-}pip)]$·HCl and *trans*-$[PtCl_2(NBA)(pip\text{-}pip)]$·HCl were selected for in vivo studies. Acute and chronic toxicity of cisplatin and acute and chronic toxicity of the novel transplatinum complexes were checked on two types of mice:

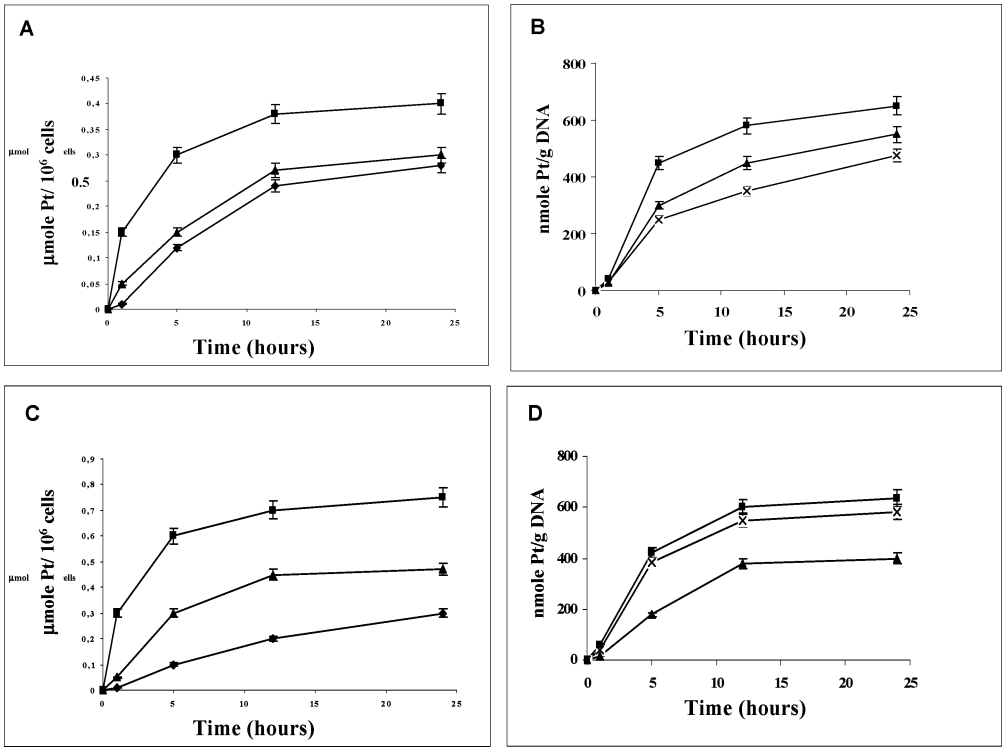


Figure 3. Platinum accumulation and DNA binding of the *trans*-Pt(II) complexes as measured by TXRF: (A) cellular accumulation in 41McisR cells; (B) DNA platination in 41McisR cells; (C) cellular accumulation in A2780cisR cells; (D) DNA platination in A2780cisR cells.

Table 2. IC_{50} Mean Values Obtained for Compounds **I** and **VI** against Ovarian Cancer Cells after 72 h of Incubation

	$IC_{50} \pm$ SD (μM) after 72 h of incubation		
complex	A2780	A2780cisR	OVCAR-3cisR
trans-[$PtCl_2(NH_3)$(pip-pip)]·HCl in NaCl (0.9%)	9 ± 2	30 ± 3.5 (3.3)	13 ± 2
trans-[$PtCl_2$(NBA)(pip-pip)]·HCl in dextrose 5%	3.7 ± 0.58	12.5 ± 1 (3.4)	not done
trans-[$PtCl_2(NH_3)$(pip-pip)]·HCl in NaCl (0.9%)	1.7 ± 0.2	2.5 ± 0.3 (1.5)	3 ± 0.5
trans-[$PtCl_2NH_3$(pip-pip)]·HCl/dextrose 5%	0.8 ± 0.5	1.5 ± 0.3 (1.9)	not done
cisplatin in NaCl (0.9%)	0.1 ± 0.05	2 ± 1 (20)	6.3 ± 1

8-week-old female BALB/c mice or on 8- to 9-week-old BALB/c nude mice. The complexes, at different concentrations, were injected iv three times at weekly intervals, and animal weight and survival were evaluated. Toxicity studies show that both *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and *trans*-[$PtCl_2$(NBA)-(pip-pip)]·HCl were nontoxic at 20 mg/kg while cisplatin could only be used at a dose of 5 mg/kg. The antitumor efficacy of the compounds was evaluated in mice bearing A2780 and A2780cisR tumors as detailed in the Experimental Section. Briefly, female BALB/c nude mice were injected ip with 5×10^6 A2780 cells or with 5×10^6 A2780cisR cells. To determine the therapeutic efficacy, cisplatin (5 mg/kg) or *trans*-[$PtCl_2$-(NH_3)(pip-pip)]·HCl or *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl (20 mg/kg) were injected iv. Treatment began on day 7 after tumor inoculation and was repeated twice for a total of three injections at 7-day intervals. The nontreated animals served as the control. Each group was composed of eight mice. The median survival and % ILS (percentage increase in life span) of treated (T) over control (C) animals [$(T \times 100/C) - 100$] were calculated. The results appear in Figure 4 and in Table 3. Cisplatin was more efficacious than both *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl in the A2780 tumor model, consistent with its lower IC_{50} values in cell cultures (Figure 4A). Cisplatin increased the life span of the mice by 160%, *trans*-

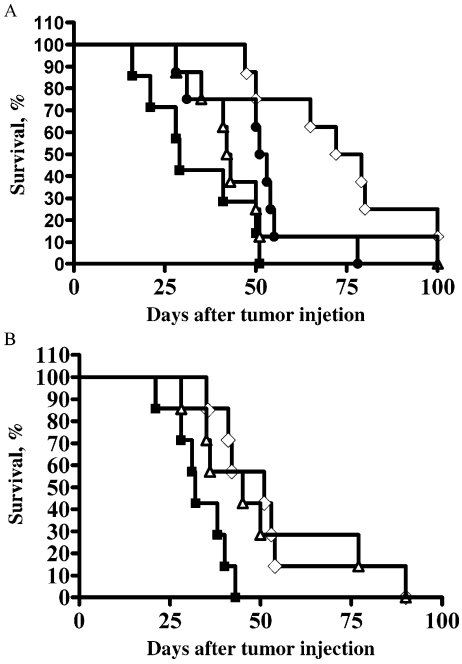


Figure 4. (A) Therapeutic activity of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl (circles), *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl (triangles), and cisplatin (diamonds) compared to that of the untreated group (squares) against cisplatin sensitive tumor. The percent survival of BALB/c nude mice inoculated ip with 5×10^6 A2780 ovarian carcinomas is shown, and the treatment is as described in Experimental Section. (B) Therapeutic activity of *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl (triangles) and cisplatin (diamonds) compared to that of untreated group (squares) against cisplatin-resistant tumor. The percent survival of BALB/c nude mice inoculated ip with 5×10^6 A2780cisR ovarian carcinomas is shown, and the treatment is as described in Experimental Section.

[$PtCl_2(NH_3)$(pip-pip)]·HCl by 79%, and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl by 46%. In the resistant A2780cisR cell line, the results obtained in vivo did not correlate with the in vitro

Table 3. Results of the in Vivo Efficacy Studies with Ovarian Cancer Cells

	compd	median survival time of A2780 cells (day)	ILS (%)	median survival time of A2780cisR cells (day)	ILS (%)
I	*trans*-[$PtCl_2(NH_3)$-(pip-pip)]·HCl	52[a]	79[a]	32[a]	0
II	*trans*-[$PtCl_2$(NBA)-(pip-pip)]·HCl	42[a]	46[a]	45[a]	40[a]
	cis-$PtCl_2(NH_3)_2$	75[a]	160[a]	51[a]	59[a]
	control	29		32	

[a] Statistically significant.

Table 4. IC_{50} Mean Values, Cellular Accumulation, and DNA Platination in C-26 Cancer Cells after 24 h of Incubation

	IC_{50} (μM)	cell accumulation (pmol of Pt per 1×10^6 cells)	Pt−DNA adducts (pmol of Pt per 50 μg of DNA)
cisplatin	2.5 ± 0.2	2244 ± 754	149 ± 22
trans-[$PtCl_2(NH_3)$(pip-pip)]·HCl	5.8 ± 0.6	54461 ± 2876	297 ± 31
trans-[$PtCl_2$(NBA)(pip-pip)]·HCl	4.5 ± 0.9	21065 ± 1955	197 ± 40
transplatin	46 ± 3.1	384 ± 122	0

cytotoxicity studies. In the in vivo studies cisplatin was more efficacious than *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl with ILS values of 59%, 0%, and 40%, respectively (Figure 4B). Interestingly, both transplatinum compounds showed similar IC_{50} values, and yet the most cytotoxic compound in A2780cisR cells, *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl, had no efficacy in vivo while treatment with *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl significantly prolonged the life span of the treated animals (ILS of 40%).

4. Studies with Colon Cancer Cells. 4.1. Cytotoxicity, Cell Accumulation, and Nuclear DNA Binding Studies. Cisplatin, *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl, and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl were screened in vitro against murine colon cancer cells C-26. The IC_{50} values were measured by the MB method as previously described.[10] The results of the 24 h incubation with the compounds appear in Table 4. Both compounds are significantly more potent than transplatin but slightly less potent than cisplatin. In contrast with the cytotoxicity studies in the ovarian cancer cells lines, in this case there was no real difference between the potencies of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl, which have IC_{50} values of 5.8 ± 0.6 and 4.5 ± 0.9 μM, respectively.

Cell accumulation and the levels of platination of cellular DNA were measured as previously described.[10] *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl accumulated in the C-26 cells 25 times more efficiently than cisplatin and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl nearly 10 times more efficiently than cisplatin. As for nuclear DNA platination, *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl was only twice as efficient as cisplatin while *trans*-[$PtCl_2$(NBA)-(pip-pip)]·HCl was only 33% more efficient than cisplatin. Neither the efficiency of cell accumulation nor that of DNA platination levels correlated with the cytotoxicity. It is noteworthy that a higher percentage of the cell-associated cisplatin ended up on the DNA compared with both trans pip-pip complexes. This result might be due to more efficient cytosolic detoxification of the pip-pip complexes or to more efficient DNA repair of these complexes or to both (see following paper).[28]

4.2. In Vivo Studies in C-26 Cancer Tumor-Bearing Mice. Acute and chronic toxicity of cisplatin and acute and chronic toxicity of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl were checked on mice as described. The toxicity studies showed that *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl was nontoxic at 20 mg/kg while cisplatin could only be used at a dose of 5 mg/kg. The antitumor activity of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl was evaluated in mice bearing C-26 tumors. Upon treatment of mice according to the schedule described (see Experimental Section), it was found that *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl significantly prolonged the life span of the treated animals (ILS = 104%) and the effect was comparable to that of cisplatin (ILS = 118%) (Figure 5). Median survival time of mice treated with *trans*-[$PtCl_2(NH_3)$-(pip-pip)]·HCl (**I**) was 22.5 days ($p < 0.0001$) compared with 24 days in the case of treatment by cisplatin ($p < 0.0001$) and 11 days of survival of the control (untreated) group. The in vivo efficacy results correlated with the results obtained in the in vitro cytotoxicity studies where cisplatin and *trans*-[$PtCl_2(NH_3)$-(pip-pip)]·HCl had comparable efficacies.

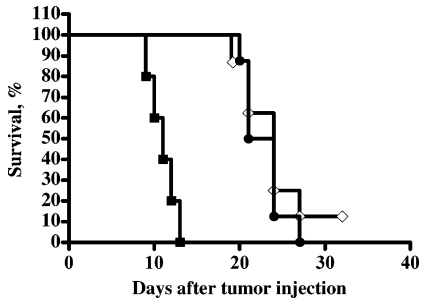


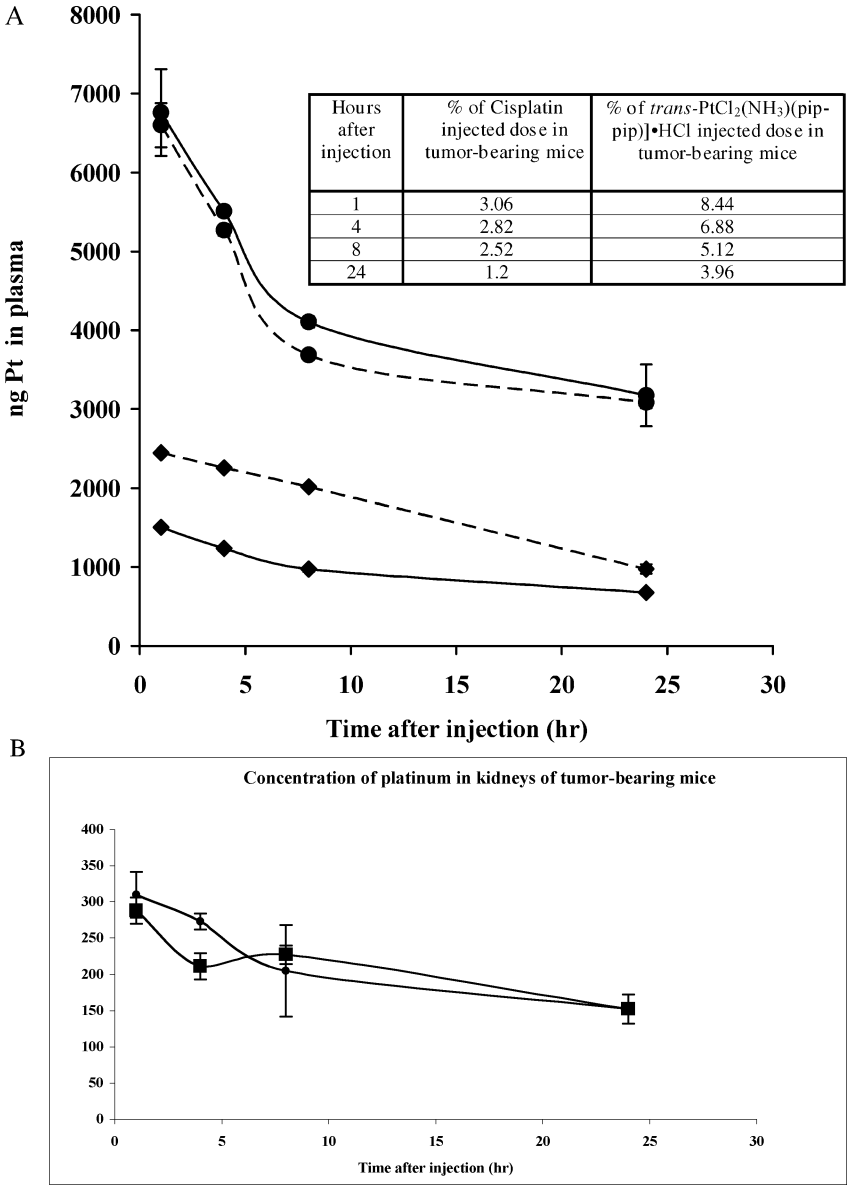
Figure 5. Therapeutic activity of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl (circles) and cisplatin (diamonds) compared to that of untreated group (squares). The percent survival of BALB/c mice inoculated ip with 1×10^6 C-26 colon carcinomas is shown, and the treatment is as described in Experimental Section.

Preliminary pharmacokinetic studies were performed. Total Pt concentrations in the blood of normal and tumor-bearing female BALB/c mice injected with cisplatin or *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl were performed. In addition, the Pt levels in the kidneys were measured in tumor-bearing mice. The Pt levels were measured by ICP-MS**.** The plasma concentrations of trans-[$PtCl_2(NH_3)$(pip-pip)]·HCl were about 2.5-fold greater than those of cisplatin at all time periods after their injection. Also, there was no significant difference in the Pt concentrations of the two compounds in the plasma of normal vs tumor-bearing mice (Figure 6A) and in the kidneys of tumor-bearing mice, indicating their similar clearance rate from plasma by kidneys (Figure 6B).

Discussion

Aiming to expand the structure−activity relationship of novel trans oriented platinum complexes, we have synthesized and characterized series of compounds based on the nonplanar heterocyclic ligand 4-piperidinopiperidine (pip-pip). All these compounds have a positive charge that is removed from the metal center. The rationale behind the design of these compounds was to enhance the water solubility of the platinum complexes and to form DNA adducts different from those formed by cisplatin and transplatin. Toward this end we incorporated a ligand (pip-pip) that is cationic, nonplanar, and semiflexible so that it can interact with the double helix of the DNA, and then we systematically varied the structural features of the amine ligand trans to it.

In vitro cytotoxicity studies were performed against several cancer cell lines, both cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines and for comparison against colon cancer cells. The results indicated that replacing one ammine ligand of transplatin with pip-pip markedly increases the cytotoxicity relative to transplatin, indicating that a positively charged nonplanar amine ligand activates the trans geometry. The most



Hours after injection	% of Cisplatin injected dose in tumor-bearing mice	% of *trans*-$PtCl_2(NH_3)$(pip-pip)]•HCl injected dose in tumor-bearing mice
1	3.06	8.44
4	2.82	6.88
8	2.52	5.12
24	1.2	3.96

Figure 6. Pharmacokinetics (A) and clearance by kidneys (B) of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl (circles) and cisplatin (diamonds) in normal (black) and tumor-bearing mice (gray) as described in Experimental Section. Three mice per group were used.

striking results in the cytotoxicity studies against the ovarian cancer cell lines are the ability of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl to overcome the acquired resistance to cisplatin in all three resistant cell lines and the large difference in the cytotoxic properties between *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and *trans*-[$PtCl_2(CH_3NH_2)$(pip-pip)]·HCl that only differ by a CH_2 group. To try and gain further insights into the determinants of cytotoxicity, the cellular accumulation and DNA platination in 41McisR and A2780cisR cells were measured. While the cell accumulation and DNA platination levels corresponded to the IC_{50} values, it is hard to rationalize why in the 41McisR cells (resistant by reduced accumulation) cisplatin, which does not have even a single carbon atom, should accumulate as efficiently as *trans*-[$PtCl_2(CH_3NH_2)$(pip-pip)]·HCl or why *trans*-[$PtCl_2(CH_3NH_2)$(pip-pip)]·HCl accumulates less efficiently than *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl. It should, however, be kept in mind that cellular accumulation experiments cannot distinguish between cytosolic platinum and membrane-bound platinum, and hence, the results do not necessarily reflect the actual cytosolic concentration of the Pt complex. DNA platination levels reflect the balance between several processes such as cytosolic accumulation, interactions with intracellular platinophiles, binding rates to DNA, and differential DNA repair efficiencies, and hence, it is impossible to single out one factor that can be altered in order to increase DNA binding levels.

The complexes used in this study differ in lipophilicity (as indicated by the % C of the compounds, which increased from 25% to 32% on going from compound **I** to compound **VII**(**I**)) and in the steric hindrance of the aliphatic amines trans to the pip-pip ligand. Lipophilicity can affect cellular accumulation and hence the cytotoxicity, while steric hindrance can affect the cytotoxicity by reducing the reactivity of the complex toward undesirable nucleophiles or altering the DNA binding properties. There were no clear correlations between the lipophilicity and the cytotoxicity as the Am ligand changes from ammine through methylamine and dimethylamine to propylamine, butylamine, and cyclohexylamine. This was true for all six ovarian cancer cell lines. The two complexes with the largest steric crowding around the Pt center are *trans*-[$PtCl_2$(DMA)(pip-pip)]·HCl and *trans*-[$PtCl_2$(IPA)(pip-pip)]·HCl. Their cytotoxic properties were not significantly different from their less hindered analogues *trans*-[$PtCl_2$(MA)(pip-pip)]·HCl and *trans*-[$PtCl_2$(NPA)(pip-pip)]·HCl. These results exemplify the difficulty of rationally designing a cytotoxic platinum complex and reinforce the need

to continue the preparation and evaluation of novel complexes in the search for improved drugs.

In the case of the colon cancer cells, the pattern was different. The cellular accumulation of cisplatin is about 10- or 25-fold lower than for *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl and *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl, respectively. Despite the more efficient uptake of the cationic complexes and the significantly faster DNA binding,[28] the DNA platination levels of the cationic complexes were comparable to those of cisplatin. *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl formed only 3-fold more DNA adducts than cisplatin even though its cellular accumulation was 25-fold higher than cisplatin. This may be due in part to the more efficient interactions of the pip-pip complexes with GSH.[28] In contrast to the ovarian cancer cells, in the colon cancer cells there seems to be an inverse correlation between the DNA platination levels and the IC_{50} values.

There have been many publications describing the in vitro cytotoxicity of transplatinum compounds, but fewer in vivo studies have been reported.[23−26] The *trans*-Pt pip-pip complexes are significantly less toxic than cisplatin. In the ovarian cancer xenografts with the sensitive cell line A2780, the in vivo efficacy reflected the pattern of the in vitro cytotoxicity results: cisplatin > *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl > *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl. Yet, in the in vitro studies cisplatin was 10 times more potent than *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and 7 times more potent than *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl, while in the in vivo studies it was only twice as efficacious as *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and 3.5 more efficacious than *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl. In the C-26 xenografts *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl was as efficacious as cisplatin. In the case of the C-26 cell line there was a correlation between the in vitro cytotoxicity and in vivo efficacy.

The pharmacokinetics studies demonstrated that there was no significant difference in concentrations of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl and of cisplatin in the plasma of normal or tumor-bearing mice and the clearance of both drugs from plasma was the same. Therefore, we suggest that the inferior therapeutic activity of *trans*-Pt based pip-pip complexes might be due to more efficient cytosolic detoxification of the pip-pip complexes or to more efficient DNA repair of these complexes or to both.

In previous studies we described platinum compounds that have either the neutral piperidine (pip) or cationic piperazine (pz) heterocyclic ligand trans to the ammine group. Together with compound **I** all these complexes represent nonsymmetric transplatinum complexes, with a heterocyclic nonplanar ligand trans to the NH_3 group. *trans*-[$PtCl_2$(pip)(pz)]·HCl is cationic and has two nonplanar heterocyclic amine ligands and may be considered the symmetric analogue of *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl. With the exception of the A2780 cell line, the nonsymmetric complex is significantly more potent than the symmetric one especially in the resistant cell lines where the *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl is 2.6, 5.6, and 2.4 times more potent than *trans*-[$PtCl_2$(pip)(pz)]·HCl against A2780cisR, CH1cisR, and 41McisR, respectively.

Replacing a monocyclic cationic amine (pz) with a bicyclic cationic amine (pip-pip) affected the in vitro potencies of the trans compounds. *trans*-[$PtCl_2$(NBA)(pz)]·HCl and *trans*-[$PtCl_2$(NBA)(pip-pip)]·HCl have similar potencies in the three sensitive cell lines and in the A2780cisR, but the pz complex is 3.7 and 2.8 times more potent than the pip-pip complex in CH1cisR and 41McisR, respectively. *trans*-[$PtCl_2(NH_3)$(pz)]·HCl and *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl show essentially the opposite picture where the pip-pip complex is 1.8, 2.2, and 3.2 times more potent than the pz complex against A2780cisR, CH1cisR, and 41McisR.

Experimental Section

Materials. Potassium tetrachloroplatinate (K_2PtCl_4), 4-piperidinopiperidine (pip-pip), methylamine (MA), dimethylamine (DMA), *n*-butylamine (NBA), isopropylamine (IPA), cyclcohexylamine (CHA), and *cis*- and *trans*-DDP were all purchased from Sigma-Aldrich Israel Ltd. and were used without further purification.

^{195}Pt NMR Spectroscopy. All platinum complexes synthesized were characterized by ^{195}Pt NMR spectroscopy. Data were collected on a Varian Unity Inova 500 MHz spectrometer equipped with a 5-mm switchable probe. The platinum chemical shifts were measured relative to the external reference signal of K_2PtCl_4, set at −1624 ppm. A line broadening of 300 Hz was normally applied, and data were processed using the VNMR software.

ESIMS. ESIMS was performed using a ThermoQuest Finnigan LCQ-Duo in the positive ion mode. Elution was in a mixture of 49:49:2 water/methanol/acetic acid at a flow rate of 15 μL/min.[27]

ICP-MS. ICP-MS measurements were performed on a Elan-DRC II, Perkin-Elmer Sciex spectrometer. The platinum concentration was calculated according to a known concentration of a K_2PtCl_4 standard solution (10 ng/mL Pt).

Total X-ray Fluorescence Measurements (TXRF). The analysis by total reflection X-ray fluorescence of the intracellular platinum content or platinum bound to nuclear DNA was performed using a Extra-II spectrometer (Seifert, Ahrensburg, Germany) according to a procedure previously reported.[26]

Synthesis. The general procedures for preparing *trans*-[$PtCl_2$(Am)(pip-pip)]·HCl, Am = NH_3, MA, DMA, IPA, NPA, NBA, CHA are as follows.

Synthesis of the Intermediate *cis*-[PtI2(Am)$_2$]. Potassium tetrachloroplatinate, K_2PtCl_4 (1.0 g), was dissolved in 25 mL of DDW, and an amount of 8 equiv (3.2 g) of KI was added. The mixture was stirred at room temperature for 30 min. Two equivalents of the relevant amine (Am) were added, and the mixture was vigorously stirred for 2−3 h at room temperature during which a yellow precipitate of the *cis*-diiododiamineplatinum(II) was formed. The yellow precipitate was collected by filtration, washed with 50 mL of DDW, and dried by washing with diethyl ether. The yields for the reactions were in the range 85−95%. All compounds were characterized using ^{195}Pt NMR and showed resonances in the δ −3310 to −3330 ppm range.

Synthesis of the Tetraam(m)ine Intermediate *cis*-[Pt(pip-pip)$_2$(Am)$_2$]$^{2+}$. In the dark, *cis*-[$PtI_2(Am)_2$] was dissolved in 50 mL of DMF, an amount of 2 equiv of $AgNO_3$ was added, and the mixture was stirred for 24 h at room temperature. By the end of the reaction the yellow precipitate was filtered off through Celite. The filtrate was collected. An amount of 2 equiv of the ligand (pip-pip) was added, and the solution was heated to 60 °C for 2−3 h (in some cases a black precipitate formed). The reaction was stopped when the ^{195}Pt NMR displayed a single resonance for the tetraam(m)ine complex in the range δ −2600 to −2700 ppm. The tetraamines, *cis*-[$Pt(pip\text{-}pip)_2(Am)_2]^{2+}$, were not isolated but used for the subsequent reaction.

Synthesis of the *trans*-[PtCl$_2$(pip-pip)(Am)]·HCl. The reaction mixture was cooled to room temperature and filtered, and the DMF was evaporated to dryness under reduced pressure. The resulting gum was dissolved in 20 mL of DDW to which an amount of 2 mL of concentrated HCl was added. The temperature was elevated to reflux (∼100 °C) for several hours depending on the amine (for each amine a different time was required; see below). The reaction time was adjusted by monitoring with ^{195}Pt NMR. After the reaction ended, the solution was chilled at 0 °C for 24 h, affording the yellow precipitate of *trans*-[$PtCl_2$(Am)(pip-pip)]·HCl. In some cases the product was obtained by allowing the aqueous acidic reaction mixture to stand at room temperature in an open reaction vessel for slow evaporation of the solvent. The precipitate was filtered, washed with 30 mL of cold DDW, and dried with diethyl ether.

***trans*-[$PtCl_2$(pip-pip)(NH_3)]·HCl (I).** Reflux time in acidic solution was 4 h. Yield 78%. Anal. Calcd for $C_{10}H_{24}Cl_3N_3Pt$: C, 24.35%; H, 5.00%; N, 8.82%. Found: C, 25.06%; H, 3.82%; N, 8.55%. ^{195}Pt NMR(H_2O): $\delta = -2220$ ppm. ESI-MS *m*/*z*: 450.09, 451.09, 452.08, 453.08, 454.07.

***trans*-[$PtCl_2$(pip-pip)(MA)]·HCl (II).** Reflux time in acidic solution was 4.5 h. Yield 85%. Anal. Calcd for $C_{11}H_{26}Cl_3N_3Pt$: C, 26.33%; H, 5.22%; N, 8.37%. Found: C, 26.00%; H, 5.20%; N, 7.82%. ^{195}Pt NMR(H_2O): $\delta = -2221$ ppm. ESI-MS *m*/*z*: 464.04, 465.11, 466.06, 467.17, 468.2, 469.16.

***trans*-[$PtCl_2$(pip-pip)(DMA)]·HCl (III).** Reflux time in HCl was 4.5 h. Yield 35%. Anal. Calcd for $C_{12}H_{32}Cl_3N_3O_2Pt$: C, 26.12%; H, 5.84%; N, 7.16%. Found: C, 25.85%; H, 6.16%; N, 8.72%. ^{195}Pt NMR(H_2O): $\delta = -2200$ ppm.

***trans*-[$PtCl_2$(pip-pip)(IPA)]·HCl (IV).** Reflux time in HCl was 5 h. Yield 35%. Anal. Calcd for $C_{13}H_{30}Cl_3N_3Pt$: C, 28.49%; H, 5.90%; N, 7.11%. Found: C, 25.06%; H, 3.82%; N, 8.55%. ^{195}Pt NMR(H_2O): $\delta = -2226$ ppm. ESI-MS *m*/*z*: 491.8, 492.07, 493.13, 495.87, 496.2.

***trans*-[$PtCl_2$(pip-pip)(NPA)]·HCl (V).** Reflux time in HCl was 5 h. Yield 70%. Anal. Calcd for $C_{13}H_{30}Cl_3N_3Pt$: C, 28.49%; H, 5.90%; N, 7.11%. Found: C, 24.77%; H, 5.66%; N, 6.57%. ^{195}Pt NMR(H_2O): $\delta = -2224$ ppm. ESI-MS *m*/*z*: 493.1, 494.07, 496.09.

***trans*-[$PtCl_2$(pip-pip)(NBA)]·HCl (VI).** Reflux time in HCl was 4.5 h. Yield 75%. Anal. Calcd for $C_{14}H_{32}Cl_3N_3Pt$: C, 30.92%; H, 5.93%; N, 7.73%. Found: C, 29.81%; H, 6.00%; N, 7.01%. ^{195}Pt NMR(H_2O): $\delta = -2224$ ppm. ESI-MS *m*/*z*: 506.13, 07.07, 508, 509.07, 510.13.

***trans*-[$PtCl_2$(pip-pip)(CHA)]·HCl·H_2O (VII).** Reflux time in HCl was 5 h. Yield 70%. Anal. Calcd for $C_{16}H_{36}Cl_3N_3OPt$: C, 32.69%; H, 6.17%; N, 7.15%. Found: C, 31.82%; H, 5.77%; N, 7.33%. ^{195}Pt NMR(H_2O): $\delta = -2221$ ppm. ESI-MS *m*/*z*: 532.1, 533.16, 534.13, 535.13, 536.04.

Cytotoxicity Studies. Biological Reagents. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma Co. (Alcobendas, Spain). The platinum compounds were dissolved in 10 mM $NaClO_4$ in distilled water. Stock solutions of the compounds at 1 mg/mL were freshly prepared before use.

Culture Conditions Used for Ovarian Cancer Cell Lines. Cultures of pairs of cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines (A2780/A2780cisR, 41M/41McisR, and CH1/CH1cisR normal) have been described elsewhere.[19] These pairs of cell lines were selected on the basis of encompassing all of the known major mechanisms of resistance to cisplatin: 41McisR being resistant primarily through reduced drug transport,[20] CH1cisR through enhanced DNA repair/tolerance,[21] and A2780cisR through a combination of decreased uptake, enhanced DNA repair/tolerance, and elevated GSH levels. [22]

Culture Conditions Used for the Colon Cancer Cell Line. Murine colon carcinoma cell line (C-26) was maintained in RPMI-1640 medium supplemented with 10% FCS, antibiotics, and glutamine. Human ovarian cancer cisplatin-sensitive A2780 and cisplatin-resistant A2780cisR cell lines and the OVCAR-3 cisR cell line were maintained in DMEM medium supplemented with 10% FCS, antibiotics, and glutamine. All culture medium components were purchased from Biological Industries (Beit-HaEmek, Israel). All cell lines were maintained at 37 °C in a water-jacketed CO_2 incubator.

Cytotoxicity Measurements in Ovarian Cancer Cells. Cell survival in compound-treated cultures was evaluated by the MTT method as previously reported. Platinum compounds were added to 96-microwell plates containing the cell cultures at final concentrations between 0 and 200 μM. After 24 h, cell survival was evaluated by measuring the absorbance at 520 nm, using a Whittaker microplate reader 2001. IC_{50} values (compound concentration that produces 50% of cell killing) were calculated from curves constructed by plotting cell survival (%) versus compound concentration (μM). Experiments were carried out in quadruplicate.

Cytotoxicity Measurements in C-26 Cancer Cells. The cytotoxicity of transplatinum compounds was tested by the methylene blue (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, 4 wells were used. Following 24 h of incubation in culture, an amount of 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 the 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 non-cell-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.

In Vivo Evaluation of Toxicity and Antitumor Efficacy. All the experimental procedures that make use of animals (mice) were done in accordance with the standards required by the Institutional Animal Care and Use Committee of the Hebrew University and Hadassah Medical Organization and approved by the Committee. Acute and chronic toxicity of novel transplatinum complexes was checked on two types of mice: 8-week-old female BALB/c mice or 8- to 9-week-old BALB/c nude mice and compared to cisplatin. These novel complexes, at different concentrations, were injected iv three times at weekly intervals, and animal weight and survival were evaluated.

Female BALB/ c mice (in the weight range 17−20 g) were injected ip with 1×10^6 of murine C-26 colon carcinomas. The viability of these cells was >90% by trypan blue exclusion. To determine the therapeutic efficacy of the novel compounds, the 5 mg/kg of *trans*-[$PtCl_2$(NH_3)(pip-pip]·HCl or 5 mg/kg of cisplatin were injected iv. Treatment began on day 4 after tumor inoculation and was repeated twice for a total of three injections at 7-day intervals. The nontreated animals served as the control. Each group was composed of eight mice. The median survival and % ILS (percentage increase in life span) of treated (*T*) over control (*C*) animals, ($T \times 100/C$) − 100, were calculated.

Female BALB/c OlaHsd nude mice (in the weight range 17−20 g) were injected ip with 5×10^6 of human A2780 cisplatin-sensitive ovarian cancer cell line (A2780S) or with 5×10^6 of human A2780 cisplatin-resistant ovarian cancer cell line (A2780R). To determine the therapeutic efficacy of the novel compounds, the 20 mg/kg of *trans*-[$PtCl_2$(NH_3)(4-piperidinopiperidine)]·HCl or of *trans*-[$PtCl_2$-(NBA)(4-piperidinopiperidine)]·HCl were injected iv and compared to cisplatin (5 mg/kg). Treatment began on day 7 after tumor inoculation and was repeated twice for a total of three injections at 7-day intervals. The nontreated animals served as the control. Each group was composed of eight mice. The median survival and % ILS (percentage increase in life span) of treated (*T*) over control (*C*) animals, ($T \times 100/C$) − 100, were calculated.

Pharmacokinetic Studies in Normal and C-26 Tumor-Bearing Mice. To measure total Pt concentration in the blood of normal and tumor-bearing mice, cisplatin and *trans*-[$PtCl_2NH_3$(pip-pip)]·HCl were dissolved in 0.9% NaCl and injected at a dose of 5 mg/kg iv into female BALB/c mice (in the weight range 17−20 g). Blood samples were immediately placed on ice and centrifuged at 14 000 rpm for 5 min at 4 °C, and the plasma was collected and stored at 80 °C until platinum analysis. For the analysis the blood samples were mineralized overnight in 65% HNO_3 (BDH, England). Before analysis, samples were dissolved in deionized water 60-fold and measured by ICP-MS. For measurements of total Pt concentration in kidneys of tumor-bearing mice, 100 mg of frozen kidney was extracted overnight with 0.5 mL of benzethonium hydroxide (Sigma, Swijndrecht, The Netherlands) and diluted with

4.25 mL of 0.2 M HCl. Before analysis, samples were dissolved 320-fold and measured by ICP-MS.

Statistical Analysis. Median survival times and the statistical significance of differences in survival curves were calculated by means of the log-rank test using Prism Software (GraphPad, San Diego, CA). Differences were considered significant at $p < 0.05$.

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Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rosenberg, B.; Vancamp, L.; Krigas, T. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature* **1965**, *205*, 698−699.
(2) Wong, E.; Giandomenico, C. M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **1999**, *99*, 2451−2466.
(3) Boulikas, T.; Vougiouka, M. Cisplatin and platinum drugs at the molecular level. *Oncol. Rep.* **2003**, *10*, 1663−1682.
(4) 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*, 87−210.
(5) 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.
(6) (a) Natile, G.; Coluccia, M. Antitumor Active Trans-platinum Compounds. In *Metal Complexes in Tumor Diagnosis and as Anticancer Agents*; Sigel, A., Sigel, H., Eds.; Metal Ions in Biological Systems, Vol. 42; Marcel Dekker: New York, 2004; pp 209−250. (b) Natile, G.; Coluccia, M. Current status of trans-platinum compounds in cancer therapy. *Coord. Chem. Rev.* **2001**, *216*, 383−410.
(7) 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.
(8) Coluccia, M.; Nassi, A.; Loseto, F.; Boccarelli, A.; Mariggio, M. A.; Giordano, G.; Intini, F. P.; Caputo, P.; Natile, G. A transplatinum complex showing higher antitumor activity than the cis congeners. *J. Med. Chem.* **1993**, *36*, 510−512.
(9) Montero, E. I.; Diaz, S.; Gobzalez-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.
(10) Khazanov, E.; Barenholz, Y.; Gibson, D.; Najajreh, Y. Novel apoptosis-inducing trans-platinum piperidine derivatives: synthesis and biological characterization. *J. Med. Chem.* **2002**, *45*, 5196−5204.
(11) Najajreh, Y.; Perez, J. M.; Navarro-Ranninger, C.; Gibson, D. Novel soluble cationic trans-diaminedichloroplatinum(II) complexes that are active against cisplatin resistant ovarian cancer cell lines. *J. Med. Chem.* **2002**, *45*, 5189−5195.
(12) Jawbry, S.; Freikman, I.; Najajreh, Y.; Perez, J. M.; Gibson, D. Preparation, cytotoxicity and interactions with nucleophiles of three isomeric transplatinum complexes containing methylpiperidine ligands. *J. Inorg. Biochem.* **2005**, *99*, 1983−1991.
(13) Hollis, L. S.; Amundsen, A. R.; Stern, E. W. Chemical and biological properties of a new series of cis-diammineplatinum (II) antitumor agents containing three nitrogen donors: cis-[Pt-(NH_3)2(N-donor)-Cl]$^+$. *J. Med. Chem.* **1989**, *32*, 128−136.
(14) 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.
(15) Farrell, N.; Qu, Y.; Hacker, M. P. Cytotoxicity and antitumor-activity of bis(platinum) complexes. A novel class of platinum complexes active in cell-lines resistant to both cisplatin and 1,2-diaminocyclohexane complexes. *J. Med. Chem.* **1990**, *33*, 2179−2184.
(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) Perego, P.; Caserini, C.; Gatti, L.; Careninil, 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.
(18) Kasparkova, J.; Novakova, O.; Marini, V.; Najajreh, Y.; Gibson, D.; et al. Activation of trans geometry in bifunctional mononuclear platinum complexes by a piperidine ligand. Mechanistic studies on antitumor action. *J. Biol. Chem.* **2003**, *278*, 47516−47525.
(19) Kelland, L. R.; Abel, G.; McKeage, M. J.; Jones, M.; Goddard, P. M.; Valenti, M.; Murrer, B. A.; Harrap, K. R. Preclinical antitumor evaluation of bis-acetato-ammine-dichloro-cyclohexylamine platinum-(IV): an orally active platinum drug. *Cancer Res.* **1993**, *53*, 2581−2586.
(20) Loh, S. Y.; Mistry, P.; Kelland, L. R.; Abel, G.; Harrap, K. R. Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum (II) and (IV) ammine/amine complexes. *Br. J. Cancer.* **1992**, *66*, 1109−1115.
(21) Goddard, P. M.; Orr, R. M.; Valenti, M. R.; Barnard, C. F.; Murrer, B. A.; Kelland, L. R.; Harrap, K. R. Novel *trans*platinum complexes: comparative in vitro and in vivo activity against platinum-sensitive and resistant murine tumours. *Anticancer Res.* **1996**, *16*, 33−38.
(22) Behrens, B. C.; Hamilton, T. C.; Masuda, H.; Grotzinger, K. R.; Whang-Peng, J.; Louie, K. G.; Knutsen, T.; McKoy, W. M.; Young, R. C.; Ozols, R. F. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res.* **1987**, *47*, 414−418.
(23) Kelland, L. R.; Barnard, C. F.; Evans, I. G.; Murrer, B. A.; Theobald, B. R.; et al. Synthesis and in vitro and in vivo antitumor activity of a series of trans platinum antitumor complexes. *J. Med. Chem.* **1995**, *38*, 3016−3024.
(24) Goddard, P. M.; Orr, R. M.; Valenti, M. R.; Barnard, C. F.; Murrer, B. A.; et al. Novel trans platinum complexes: comparative in vitro and in vivo activity against platinum-sensitive and resistant murine tumours. *Anticancer Res* **1996**, *16*, 33−38.
(25) Coluccia, M.; Nassi, A.; Boccarelli, A.; Giordano, D.; Cardellicchio, N.; et al. In vitro and in vivo antitumour activity and cellular pharmacological properties of new platinum-iminoether complexes with different configuration at the iminoether ligands. *J. Inorg. Biochem.* **1999**, *77*, 31−35.
(26) Perez, J. M.; Kelland, L. R.; Montero, E. I.; Boxall, F. E.; Fuertes, M. A.; et al. Antitumor and cellular pharmacological properties of a novel platinum(IV) complex: trans-[$PtCl_2(OH)_2$(dimethylamine)-(isopropylamine)]. *Mol. Pharmacol.* **2003**, *63*, 933−944.
(27) Najajreh, Y.; Shulman, T. P.; Moshel, O.; Farrell, N.; Gibson, D. Ligand effects on the binding of cis- and trans- PtCl(2)Am1Am2 to proteins. *J. Biol. Inorg. Chem.* **2003**, *8*, 167−175.
(28) Najajreh, Y.Ardeli-Tzaraf, Y.; Kasparkova, J.; Heringova, P.; Prilutski, D.; Balter, L.; Jawbry, S.; Khazanov, E.; Perez, J. M.; Barenholz, Y.; Brabec, V.; Gibson, D. Interactions of platinum complexes containing cationic, bicyclic, nonplanar piperidinopiperidine ligands with biological nucleophiles. *J. Med. Chem.* **2006**, *49*, 4674−4683.

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