Mono- and Polynuclear [Alkylamine]platinum(II) Complexes of [1,2-Bis(4-fluorophenyl)ethylenediamine]platinum(II): Synthesis and Investigations on Cytotoxicity, Cellular Distribution, and DNA and Protein Binding

Timo Kapp, Anja Dullin, and Ronald Gust*

Institute of Pharmacy, Free University of Berlin, Königin Luise Strasse 2+4, 14195 Berlin, Germany

Received August 25, 2005

A series of mononuclear and dinuclear alkylamine derivatives of [*meso*-1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) (**m-4F-PtL-R$_1$** and **(m-4F-PtL)$_2$-R$_2$**; R$_1$ = alkylamine, R$_2$ = alkyldiamine, L = DMSO or Cl) as well as the DAB(PA)$_4$ polyimine dendrimer complex (**(m-4F-PtDMSO)$_4$DAB(PA)$_4$**; DAB(PA)$_4$ = *N*,*N*,*N*′,*N*′-tetrakis(3-aminopropyl)butane-1,4-diamine) were synthesized and tested for cytotoxicity, intracellular distribution, and DNA and protein binding. All compounds strongly bound to human serum albumin by hydrophobic and electrostatic interactions. These inactivation reactions hindered the uptake into tumor cells and prevented strong cytotoxic effects. If serum-free medium was used, a high accumulation grade in MCF-7 breast cancer cells and a high DNA binding was observed. As most efficient compound **(m-4F-PtDMSO)$_4$DAB(PA)$_4$** was identified. It showed a 20-fold higher cellular uptake and a ∼700-fold higher DNA binding than cisplatin.

Introduction

Platinum complexes represent important cytostatics which are used in the first-line therapy of several kinds of tumor diseases.[1] However, the therapy is not without obstacles. Besides intolerable side effects such as nephrotoxicity and myelosuppression, the major problem is the development of resistance during the therapy.[2] Unfortunately, most of the complexes investigated in clinical trials so far showed cross resistance to cisplatin. Therefore, the search for new metal based drugs is one of the main topics in experimental oncology.

In recent years polynuclear platinum complexes were identified as a very promising class of antitumor active compounds. They showed a different toxicity profile than cisplatin[3] and a slightly different mode of action; they cross-link the DNA in a 1 → 4 instead of a 1 → 2 GG-pattern, as identified for cisplatin or carboplatin. This led to a less distorted DNA structure and a reduced recognition by the DNA repair system and the HMG (high mobility group) proteins.[4,5] The binding of this kind of platinum complexes to DNA occurs in two steps: a very fast electrostatic interaction with the DNA backbone due to a positive charge of the molecule followed by coordination of DNA bases at the platinum.[6]

Interestingly, most of the molecules reached the cytoplasm of the tumor cells despite their ionic character. The trinuclear platinum complex BBR 3464 ([μ-*trans*-Pt(NH$_3$)$_2$\{*trans*-PtCl(NH$_3$)$_2$[NH$_2$(CH$_2$)$_6$NH$_2$]\}$_2$](NO$_3$)$_4$), e.g., was studied in a human osteosarcoma cell system (U2-OS) and showed a higher accumulation and DNA binding than cisplatin. In contrast to L1210 cells, whose cellular accumulation depended in a linear pattern on the drug concentration in the media, a saturation level was found for the U2-OS cells.[7,8]

The tetranuclear complex [DAB(PA-*trans*-Pt(NH$_3$)$_2$Cl)$_4$]Cl$_4$ bearing the dendrimer DAB(PA)$_4$ (*N*,*N*,*N*′,*N*′-tetrakis(3-aminopropyl)butane-1,4-diamine) as linker resulted in a less active compound although it possessed the high coulomb forces to interact with DNA.[9] It was postulated that the low cytotoxicity may be caused by transport problems across the cell membranes due to its charge and branched structure.

Furthermore, some investigations indicated a different activity of the polynuclear platinum complexes depending on the used cell line. Remarkable activity of polynuclear compounds was noticed in a wide panel of tumor cells, but for breast cancer cell lines, especially the MCF-7 cell line, a relatively low activity was found.[3,9] Therefore, we focused our attention to the design of drugs with increased selectivity for breast tumors. In this initial study, we combined the well-known [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) complex[10,11] with alkylamines, alkyldiamines, and the DAB(PA)$_4$ polyimine dendrimer. We studied the significance of the charge and the alkyl chain length of the molecules on their cytotoxicity, the cellular uptake, the nuclear content, the binding to DNA in cells, and the binding characteristics to human serum albumin (HSA).

Results and Discussion

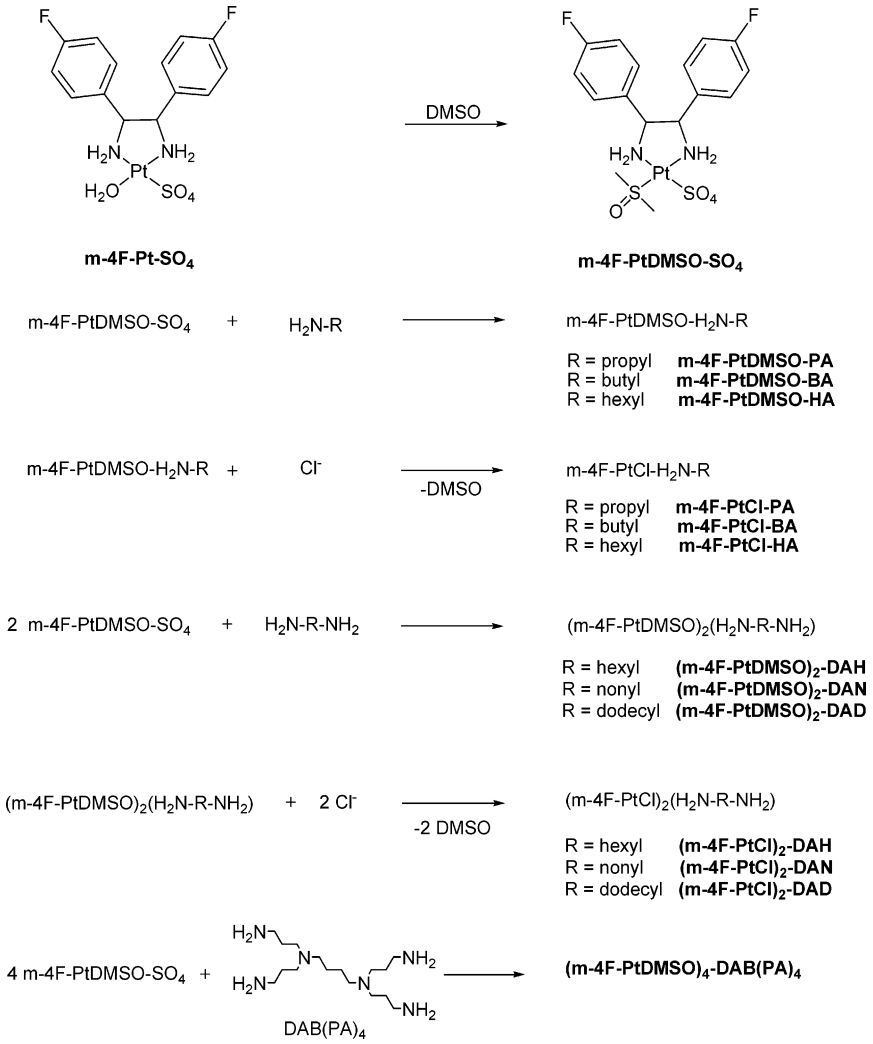
Chemistry. Synthesis. The synthesis of the [alkylamine]-platinum(II) complexes started with [*meso*-1,2-bis(4-fluorophenyl)ethylenediamine][sulfinylbis[methane]-S]sulfatoplatinum-(II) (**m-4F-PtDMSO-SO$_4$**) obtained by dissolution of the sulfatoplatinum(II) complex **m-4F-PtSO$_4$** in DMSO (see Scheme 1). Reaction of **m-4F-PtDMSO-SO$_4$** in aqueous solution with the respective alkylamine resulted in the [alkylamine][sulfinylbis[methane]-S]platinum(II) complexes (**m-4F-PtDMSO-R$_1$**; R$_1$ = alkylamine) which can finally be transformed into the [alkylamine]chloroplatinum(II) complexes **m-4F-PtCl-R$_1$** by treatment with HCl (see Scheme 1). The same reaction course can be used to synthesize the dinuclear complexes **(m-4F-PtL)$_2$-R$_2$** (R$_2$ = alkyldiamine, L = DMSO or Cl) as well as the DAB-(PA)$_4$ polyimine dendrimer complex **(m-4F-PtDMSO)$_4$DAB-(PA)$_4$** (see Scheme 1).

The characterization of the new complexes was performed by ^{1}H NMR spectroscopy. The coordination of an alkylamine to platinum is accompanied by a diastereomeric split of the amino (six signals) and the benzylic protons (two signals) due to the asymmetry of the [alkylamine][sulfinylbis[methane]-S]-platinum(II) moiety. Measurement in D$_2$O led to a complete

* To whom correspondence should be addressed. Phone: (030) 838 53272. Fax: (030) 838 56906. E-mail: rgust@zedat.fu-berlin.de.

10.1021/jm050845r CCC: $33.50
Published on Web 01/17/2006

Scheme 1. Synthesis of the [Alkylamine]platinum(II) Complexes



NH/ND exchange giving an AB system for the methine protons. The coupling constants amounted to $^3J = 5.5-6.2$ Hz, indicating for all complexes a restricted interconversion of the five-membered ethylenediamineplatinum(II) chelate ring.

The exchange of the DMSO ligand by chloride led to a collapse of the two characteristic DMSO signals at $\delta = 3.71$ and 3.74. The attempt failed to exchange all NH_2 protons by deuterium in the **m-4F-PtCl-R$_1$** complexes. If possible only the amino groups of the five-membered chelate ring allowed an NH/ND exchange by addition of D_2O.

Biological Properties. **Stability under in Vitro Conditions.** For the interpretation of the biological properties it was necessary to investigate the stability of the [alkylamine]-[sulfinylbis[methane]-S]platinum(II) complexes under in vitro conditions.

1,2-Diarylethylenediamines are stable bound to platinum in a five-membered chelate ring as already demonstrated in earlier studies.[12,13] The binding of alkylamines as realized in the mono- or dinuclear complexes seems to be much weaker. Thus substitution reactions can take place at both the Pt−alkylamine and the Pt−L bond (L = DMSO or Cl). These reactions are of high relevance for the antitumor activity as well as the toxic side effects because they allow a binding to bionucleophiles, e.g., the DNA, enzymes, or HSA.[14,15]

We investigated the reactions of the complexes in physiological NaCl solution, Delbecco's buffer, and EMEM on the example of **(m-4F-PtDMSO)$_2$-DAH** by using capillary electrophoresis (CE). This method represents a suitable and efficient analytical tool for studying the stability of platinum complexes and their reactivity toward biological relevant molecules.[16]

In physiological NaCl solution and Delbecco's buffer (Cl^- concentration: 140 mM), only an exchange of the DMSO leaving group at **(m-4F-PtDMSO)$_2$-DAH** (tm = 7.05 min) was determined, resulting in the chloroplatinum(II) derivative **(m-4F-PtCl)$_2$-DAH** (tm = 10.9 min) and the intermediate with a PtDMSO and a PtCl unit (tm = 8.35 min) (Figure 1).

Although compounds with a concentration below 5 μg/mL (detection limit) or with a low electrophoretic mobility were not detectable, an exchange of the alkylamine could be excluded. As depicted in Figure 1, the area of the peaks remained nearly constant during the experiment. In EMEM as well **(m-4F-PtDMSO)$_2$-DAH** showed exclusively a DMSO/Cl exchange. Other reaction products could not be identified.

These findings contradict our earlier results with [*meso*-1,2-bis(4-fluorophenyl)ethylenediamine]chloro[sulfinylbis[methane]-S] **(m-4F-PtDMSO-Cl)**.[17] The DMSO leaving group of **m-4F-PtDMSO-Cl** was stably bound in chloride containing medium. A cleavage as observed for other leaving group derivatives did not take place. This means that the third nitrogen of the alkylamine weakened the Pt−DMSO bond in the new compounds, and substitution reactions were made possible.

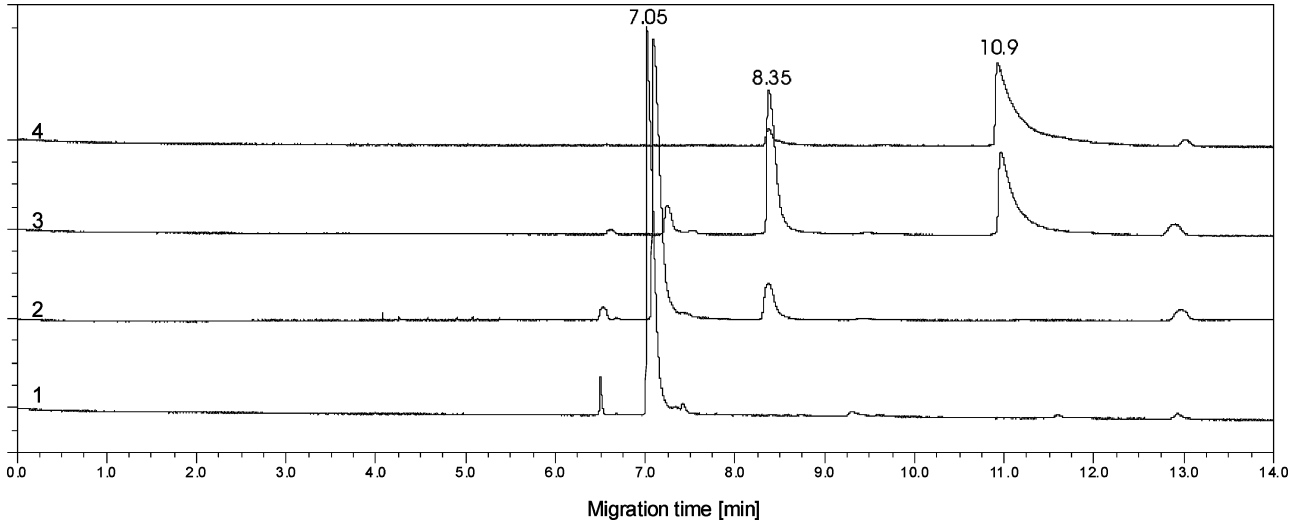


Figure 1. Electrophoretical separation of the reaction products of **(m-4F-PtDMSO)$_2$-DAH** in Delbecco's buffer: lane 1, standard **(m-4F-PtDMSO)$_2$-DAH**; lane 2, reaction mixture after 3 min; lane 3, reaction mixture after 20 min; lane 4, standard **(m-4F-PtCl)$_2$-DAH**.

This activation could explain the high cytotoxicity of **m-4F-PtDMSO-Cl** which was comparably found for cisplatin, too.[17] We assume that **m-4F-PtDMSO-Cl** hydrolyzed in the cytoplasm into the aqua(DMSO)platinum(II) **(m-4F-PtDMSO-OH$_2$)** due to the low intracellular Cl^- concentration, followed by a quick monofunctional binding to the DNA. If a cisplatin-like mode of action is realized, a destabilization of the Pt−DMSO bond by the neighboring nucleobase will be required to achieve the formation of intrastrand cross links.[18,19]

Binding to Human Serum Albumin. To evaluate whether protein binding might inactivate the complexes, all [alkylamine]-platinum(II) derivatives were incubated with HSA, and the protein unbound fraction was quantified after ultrafiltration and ethanolic precipitation by graphite furnace atomic absorption spectroscopy (GF-AAS). In contrast to the ultrafiltration, which isolates the amount of unbound drug, the ethanolic precipitation is used to determine additionally the reversible lipophilic bound drug amount.

Cisplatin, **m-4F-PtDMSO-SO$_4$**, and **m-4F-PtCl$_2$** were used as references. After ultrafiltration they respectively showed a recovery rate of 76%, 58%, and 51%. The substitution of the SO_4^{2-} leaving group in **m-4F-PtDMSO-SO$_4$** for propylamine and subsequent DMSO/Cl exchange did not influence the protein binding. However, the elongation of the alkylamine chain increased the affinity to HSA (**m-4F-PtDMSO-PA** (52%) ≈ **m-4F-PtCl-PA** (53%) < **m-4F-PtDMSO-BA** (36%) ≈ **m-4F-PtCl-BA** (43%) < **m-4F-PtDMSO-HA** (15%) ≈ **m-4F-PtCl-HA** (17%)). These data together with the high recovery of more than 70% after ethanolic precipitation (see Table 1) indicated a hydrophobic attachment at the HSA for mononuclear complexes.

The dinuclear platinum complexes bound to a much higher extent to HSA. About 0.5−10% of free platinum was determined after ultrafiltration independently of the kind of leaving group (see Table 1), and 11−16% of PtDMSO complexes as well as 35−60% of PtCl complexes were recovered after ethanolic precipitation. These data point to strong electrostatic interactions or a covalent binding to HSA.

To get an insight into the mode of action we calculated the reaction rate of irreversible protein binding. This attempt failed in the case of the PtDMSO complexes. They reached a maximum amount of free platinum after 30 min which decreased during the following incubation time. A fitting to estimate the reaction order and to determine the reaction rate was not satisfying. We assume that the PtDMSO complexes were electrostatically bound in a very fast first step. In a second step the DMSO/Cl exchange could take place, followed by a covalent binding to nucleophilic centers at the HSA.

Table 1. Protein Binding Characteristics with Human Serum Albumin[a]

compound	free amount of Pt after ultrafiltration [%]	free amount of Pt after ethanolic precipitation [%]	reaction rate for the irreversible binding to HSA $k \times 10^3$ [min^{-1}]
cisplatin	76 ± 4	100 ± 6	−5.35
m-4F-PtCl$_2$	51 ± 6	92 ± 5	−6.81
m-4F-PtDMSO-SO$_4$	58 ± 2	55 ± 2	−26.4
m-4F-PtDMSO-PA	52 ± 2	72 ± 1	nd[b]
m-4F-PtDMSO-BA	36 ± 1	92 ± 4	nd
m-4F-PtDMSO-HA	15 ± 4	101 ± 4	nd
m-4F-PtCl-PA	53 ± 3	76 ± 1	−3.13
m-4F-PtCl-BA	43 ± 4	100 ± 7	−4.56
m-4F-PtCl-HA	17 ± 1	71 ± 1	−4.84
(m-4F-PtDMSO)$_2$-DAH	8.5 ± 0.4	13 ± 1	nd
(m-4F-PtDMSO)$_2$-DAN	6.7 ± 0.1	11 ± 1	nd
(m-4F-PtDMSO)$_2$-DAD	0.5 ± 0.2	16 ± 1	nd
(m-4F-PtCl)$_2$-DAH	9.4 ± 0.7	60 ± 3	−9.51
(m-4F-PtCl)$_2$-DAN	1.1 ± 0.1	35 ± 3	−9.18
(m-4F-PtCl)$_2$-DAD	0.4 ± 0.1	36 ± 1	−8.75
(m-4F-PtDMSO)$_4$DAB(PA)$_4$	11 ± 1	23 ± 1	nd

[a] $n = 3$ for the recovery experiments. [b] nd: not determinable.

The binding of the chloro complexes followed a reaction of pseudo-first-order. The rate constants of the mononuclear complexes were somewhat lower than the one calculated for cisplatin while those of the dinuclear complexes were 2-fold higher. As an example, Figure 2 illustrates the reaction kinetic of **(m-4F-PtDMSO)$_2$-DAH** and **(m-4F-PtCl)$_2$-DAH**.

It should be noted that the platinum DAB(PA)$_4$ polyimine dendrimer conjugate **(m-4F-PtDMSO)$_4$DAB(PA)$_4$** showed as high HSA binding as the dinuclear PtDMSO complexes (11% free platinum after ultracentrifugation and 23% after ethanolic precipitation).

The interaction of platinum complexes with HSA is subject of various investigations. From the available data it can be deduced that the thiol group of the HSA molecule, namely cysteine-34, is the major site involved in cisplatin interaction.[20] Other chemically favorable platinum binding sites include the methionine-298 residue.[21] Keppler et al. investigated the binding of oxaliplatin to HSA and determined a somewhat greater binding compared to cisplatin.[22] For both compounds it is assumed that they initially coordinatively bind to HSA. This

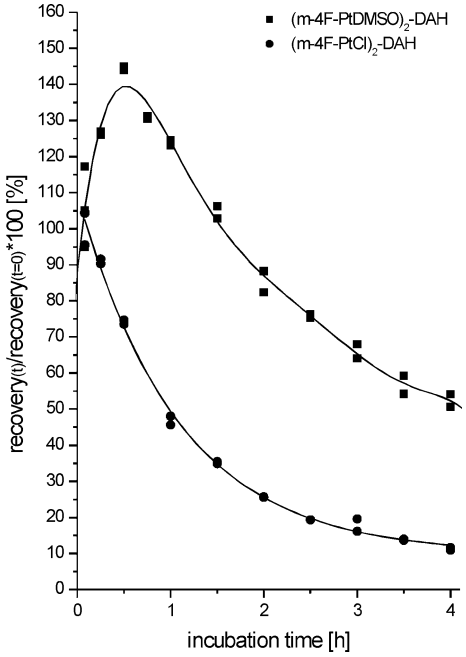


Figure 2. Decrease of free platinum after incubation of HSA with **(m-4F-PtDMSO)$_2$-DAH** and **(m-4F-PtCl)$_2$-DAH** ($n = 2$).

binding determined the kind of leaving group. The free platinum amount of Carboplatin incubated with HSA was more than 90%.

cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, being a product of cisplatin and carboplatin hydrolytic conversion in the body, exhibited a much stronger reactivity toward nuclear targets, including human serum albumin,[23] than the parent drugs. The same effect was observed for **m-4F-PtCl$_2$**, **m-4F-PtDMSO-SO$_4$**, and **m-4F-PtSO$_4$**.[11]

Cellular Uptake Studies. To quantify the accumulation grade in MCF-7 cells we incubated the cells for 24 h with the respective platinum complex in a concentration of 5 μM. The intracellular platinum content was then measured using GF-AAS. Experimental conditions were chosen to guarantee that the compounds were not inactivated by protein binding. For this purpose the cells were grown in FCS-free EMEM.

All complexes showed a time dependent accumulation grade (see Figure 3). The references cisplatin and **m-4F-PtCl$_2$** accumulated 3.7- and 26.9-fold after 24 h. The exchange of the leaving groups in **m-4F-PtCl$_2$** by DMSO and SO_4^{2-} hampered the uptake into the tumor cells. Only a 1.45-fold higher platinum content was measured for **m-4F-PtDMSO-SO$_4$** after 24 h of incubation. This might be the consequence of the positive charge of the molecule because the exchange of the SO_4^{2-} group rapidly took place in aqueous solution **(m-4F-PtDMSO-H$_2$O)**.

The exchange of the H_2O ligand in **m-4F-PtDMSO-H$_2$O** by various alkylamines enhanced the lipophilicity of the complexes without changing the molecules charge. This modification led to an increase of intracellular platinum concentration in MCF-7 cells depending on the chain length. Accumulation grade after 24 h: **m-4F-PtDMSO-PA** (2.7) < **m-4F-PtDMSO-BA** (5.5) < **m-4F-PtDMSO-HA** (43.8). The exchange of the DMSO by Cl^- reduced the charge of the complex but led only in the case of **m-4F-PtCl-HA** to a significant higher intracellular Pt-concentration (67.2-fold accumulation grade of **m-4F-PtCl-HA** after 24 h).

The linkage of two [1,2-bis(4-fluorophenyl)ethylenediamine]-platinum(II) moieties by a diaminohexane spacer reduced the uptake into the tumor cells compared to the mononuclear hexylamine complex. The accumulation grade of **(m-4F-PtCl)$_2$-DAH** and **(4F-PtDMSO)$_2$-DAH** respectively amounted to 21.5 and 30.6. Interestingly, the accumulation could be increased by elongation of the diamine spacer (**(m-4F-PtDMSO)$_2$-DAH** (21.5) < **(m-4F-PtDMSO)$_2$-DAN** (73.5) < **(m-4F-PtDMSO)$_2$-DAD** (125.6)). Analogously to the alkylamine complexes, the influence of the charge is low according to the leaving groups. The highest accumulation grade after a 24 h incubation showed **(m-4F-PtDMSO)$_2$-DAD** (125.6) and **(m-4F-PtCl)$_2$-DAD** (122.5).

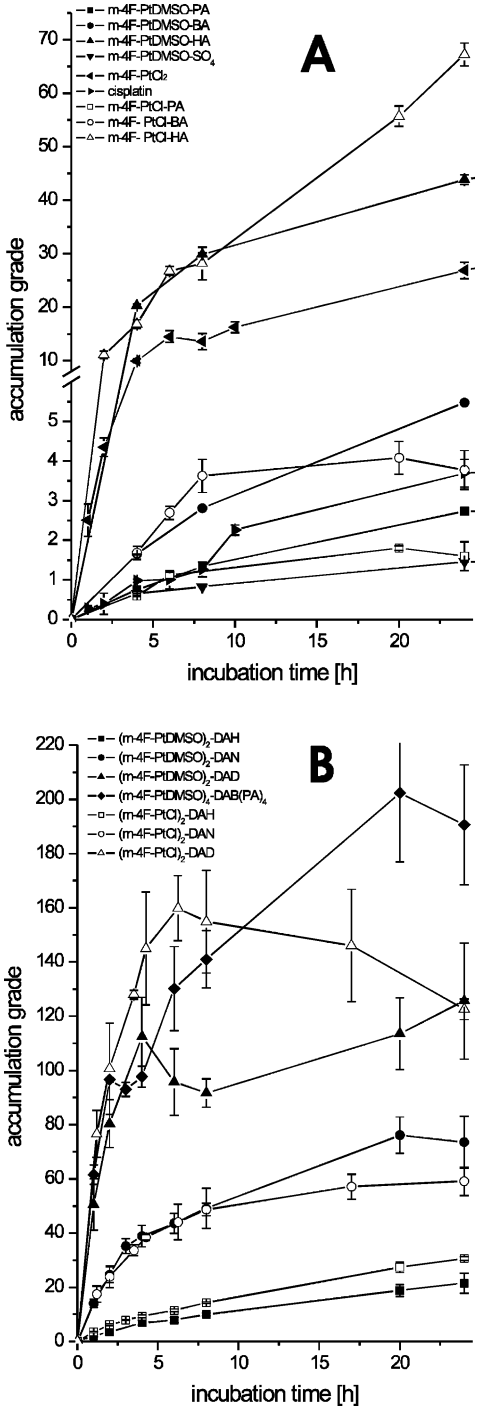


Figure 3. Cellular uptake of mononuclear (A) and polynuclear (B) [alkylamine]platinum complexes by MCF-7 cells ($n = 3$).

The comparison of the kinetic curves depicted in Figure 3 indicated for the mono- and the dinuclear complexes a fast increase of the cellular platinum concentration during 6−7 h resulting in a nearly constant saturation level. Only **(m-4F-PtCl)$_2$-DAD** showed its maximal cellular content already after

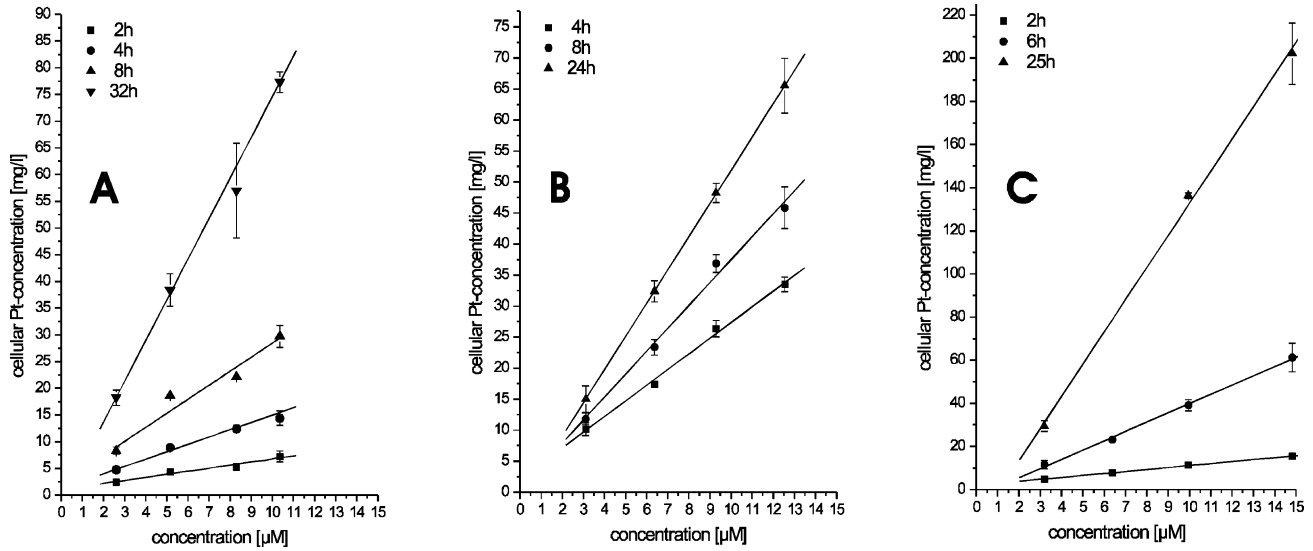


Figure 4. Dependency of intracellular platinum content on the extracellular content of different compounds (**m-4F-PtCl$_2$** (A), **m-4F-PtDMSO-HA** (B), and (**m-4F-PtDMSO)$_2$-DAH** (C)) ($n = 3$).

6 h which is then reduced by about 30% during the following 19 h of incubation.

A different accumulation curve was observed with the DAB-(PA)$_4$ dendrimer-bound platinum complex. The platinum content in the tumor cell continuously increased and reached its highest amount after a 24 h incubation. Although **(m-4F-PtDMSO)$_4$-DAB(PA)$_4$** represented a molecule with eight positive charges it was the one with the highest accumulation grade (190.7-fold after 24 h).

To get more insight into the transport mechanism through the cell membrane, we studied the validity of Fick's law on the example of **m-4F-PtCl$_2$**, **m-4F-PtDMSO-HA**, and **(m-4F-PtDMSO)$_2$-DAH**. As depicted in Figure 4 there was a dependence of the intracellular platinum amount on the drug concentration in the medium. An excellent correlation with $R^2 > 0.95$ was calculated for each of the incubation periods (up to 32 h). This means that the uncharged **m-4F-PtCl$_2$** complex as well as its cationic derivatives **m-4F-PtDMSO-HA** and **(m-4F-PtDMSO)$_2$-DAH** might be taken up into the tumor cells by a passive transport across the cell membrane. The involvement of a cation transport system such as the copper transporter as discussed for cisplatin[1,24] and its derivatives is not very likely.

Platinum Content in Nuclei and Binding to DNA. If the mononuclear and dinuclear platinum(II) complexes cause cytotoxicity due to DNA binding, it is of interest to investigate their uptake into the nuclei of tumor cells and their binding to DNA. Therefore, MCF-7 cells were incubated with the respective platinum complex (concentration: 5 μM) for 24 h, and the platinum content in the nuclei was then quantified by GF-AAS.

The nuclear platinum content achieved with cisplatin and the parent compound **m-4F-PtCl$_2$** respectively amounted to 10.0 and 165.0 ng Pt/mg protein. The exchange of the chloride leaving groups by DMSO and H_2O in **m-4F-PtCl$_2$** reduced the amount of platinum in the nuclei. Cells treated with **m-4F-PtDMSO-SO$_4$** showed 47.5 ng Pt/mg protein in the nuclei. This value decreased further by coordination of propylamine (**m-4F-PtDMSO-PA**: 6.17 ng Pt/mg protein). However, the elongation of the alkyl chain contradicted this "alkylamine effect" (see Table 2). **m-4F-PtDMSO-BA** and **m-4F-PtDMSO-HA** were taken up into the nuclei in significantly higher amounts than cisplatin but not as effective as **m-4F-PtCl$_2$** (Table 2). Interestingly, the dinuclear platinum complexes surpassed the value of **m-4F-PtCl$_2$**, dependent on the length of the diamino spacer: **(m-4F-PtDMSO)$_2$-DAH** (395 ng Pt/mg protein) < **(m-4F-PtDMSO)$_2$-DAN** (535 ng Pt/mg protein) < **(m-4F-PtDMSO)$_2$-DAD** (1184 ng Pt/mg protein). The reduction of the molecule charge enhanced the drug content in the nuclei 2- to 3-fold in each case. For **(m-4F-PtCl)$_2$-DAD** even 9924 ng Pt/mg protein was measured.

Table 2. Quantification of Platinum in the Nuclei and at the DNA of MCF-7 Cells[a]

compound	total amount of platinum in the nuclei [ng Pt/mg protein]	DNA-bound fraction [pg Pt/μg DNA]
cisplatin	10.0 ± 1.12	17.6 ± 1.0
m-4F-PtCl$_2$	165.0 ± 8.3	35.0 ± 7.2
m-4F-PtDMSO-SO$_4$	47.5 ± 1.3	4.9 ± 1.2
m-4F-PtDMSO-PA	6.17 ± 0.27	0.56 ± 0.12
m-4F-PtDMSO-BA	13.7 ± 1.67	1.84 ± 0.12
m-4F-PtDMSO-HA	60.9 ± 6.2	1.30 ± 0.53
m-4F-PtCl-PA	15.3 ± 1.1	2.39 ± 0.43
m-4F-PtCl-BA	38.3 ± 3.1	2.93 ± 0.37
m-4F-PtCl-HA	191.5 ± 17	5.61 ± 0.66
(m-4F-PtDMSO)$_2$-DAH	395 ± 55	19.6 ± 5.0
(m-4F-PtDMSO)$_2$-DAN	535 ± 56	48.6 ± 8.0
(m-4F-PtDMSO)$_2$-DAD	1184 ± 166	55.1 ± 5.3
(m-4F-PtCl)$_2$-DAH	852 ± 57	24.4 ± 6.2
(m-4F-PtCl)$_2$-DAN	1654 ± 197	78.4 ± 10.9
(m-4F-PtCl)$_2$-DAD	9924 ± 1866	89.8 ± 1.7
(m-4F-PtDMSO)$_4$-DAB(PA)$_4$	5378 ± 661	12800 ± 680

[a] $n = 3$.

The DNA-bound fraction was calculated for all complexes. For this purpose, the DNA was isolated from the nuclei, and the platinum content was measured by GF-AAS. Cisplatin was effectively bound to the DNA. The recovered platinum amounted to 17.6 pg Pt/μg DNA and correlated with data from the literature.[25] **m-4F-PtCl$_2$**, which was about 16-fold higher accumulated in the nuclei than cisplatin, showed only a 2-fold higher amount of DNA binding (35.0 pg Pt/μg DNA). This might be the consequence of steric interaction of the 1,2-bis-(4-fluorophenyl)ethylenediamine ligand during the attachment to the DNA. Cisplatin binds in a two-step reaction to the DNA. After hydrolysis, the aqua- and diaquaplatinum species attack mainly neighboring guanine bases, resulting in intrastrand cross links.[26] The hydrolysis rate of **m-4F-PtCl$_2$** (11.3×10^{-5} s^{-1}) and cisplatin (8.4×10^{-5} s^{-1}) were comparable, but the attack of nucleophilic agents was hindered due to the dynamic effects

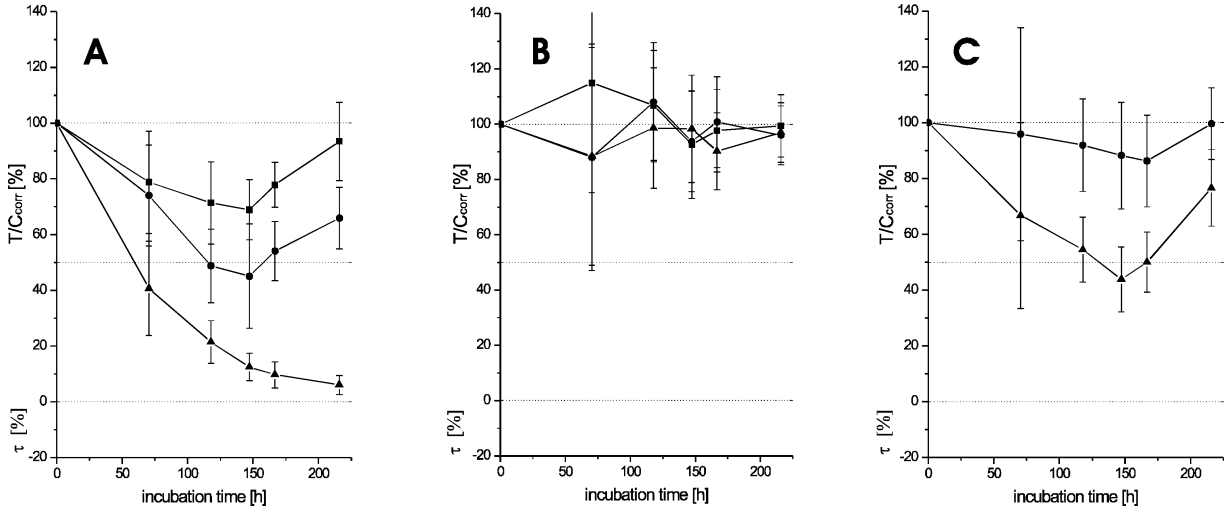


Figure 5. Cytotoxicity of cisplatin (A), (**m-4F-PtDMSO)$_2$-DAH** (B), and (**m-4F-PtDMSO)$_2$-DAB(PA)$_4$** (C) on MCF-7-cells (■, 0.5 μM; ●, 1.0 μM; ▲, 5.0 μM) (n = 16).

of the five-membered chelate ring (**m-4F-PtCl$_2$** interconverts between a λ- and a δ-conformation).[13,17] In each possible conformation at least one aryl ring is oriented out of the planar platinum plane and changed into an axial position. This aromatic ring might hinder the approach to the DNA and reduce the monofunctional binding to a nucleobase as well as the final intrastrand cross link.

The successive exchange of the chloride leaving groups in **m-4F-PtCl$_2$** by an alkylamine ligand or a DMSO leaving group drastically reduced the DNA binding (2.39 to 5.61 pg Pt/μg DNA and 0.56 to 1.30 pg Pt/μg DNA, respectively; see Table 2). The resulting complexes could only perform a monofunctional binding to the DNA (alkylamine ligands are assigned as non leaving groups), which was by far not as stable as the bifunctional coordination.

In contrast to this, dinuclear complexes are major grove binders and are able to cause interstrand cross links. This binding mode seems to be true for **(m-4F-PtDMSO)$_2$-DAH** (19.6 pg Pt/μg DNA), **(m-4F-PtDMSO)$_2$-DAN** (48.6 pg Pt/μg DNA), and **(m-4F-PtDMSO)$_2$-DAD** (55.1 pg Pt/μg DNA). The DNA platination was even higher than the one obtained with cisplatin or **m-4F-PtCl$_2$**.

The decreased content in nuclei and the low DNA binding of the PtDMSO complexes contradicted our assumption that the DMSO−Cl substitution in [alkylamine]platinum(II) complexes already took place in EMEM prior to the transport through the cell membrane. We now have to consider that both species were taken up into the tumor cells. In the cytosol the hydrolysis of the chloro complexes seemed to be faster compared to the PtDMSO complexes. As consequence a faster DNA binding occurs.

The most promising result was obtained with the dendrimer derivative **(m-4F-PtDMSO)$_4$DAB(PA)$_4$**. In the nuclei of MCF-7 cells, the platinum content amounted to 5378 ng Pt/mg protein and a DNA binding of 12800 pg Pt/μg DNA was detected, about 700-fold higher than that of cisplatin. Information about the binding mode, however, could not be given. It is very likely that ionic interaction of the highly charged molecule especially with the negatively charged phosphate backbone plays a major role.[27]

Cytotoxicity against MCF-7 Cells. To evaluate if the above-mentioned effects consequently lead to an inhibition of tumor cell growth, we investigated all [alkylamine]platinum(II) complexes for cytotoxicity at the MCF-7 cell line. The mononuclear complexes were completely inactive up to a concentration of 50 μM, while the dinuclear complexes showed antiproliferative effects with IC$_{50}$ values more than 10 times higher than cisplatin (IC$_{50}$ = 2.0 μM). In this test, the PtCl complexes (**(m-4F-PtCl)$_2$-DAH**: IC$_{50}$ = 40.4 μM; **(m-4F-PtCl)$_2$-DAN**: IC$_{50}$ = 25.2 μM; **(m-4F-PtCl)$_2$-DAD**: IC$_{50}$ = 33.5 μM) were somewhat more active than the respective PtDMSO complexes (**(m-4F-PtDMSO)$_2$-DAH**: IC$_{50}$ > 50 μM; **(m-4F-PtDMSO)$_2$-DAN**: IC$_{50}$ = 42.0 μM; **(m-4F-PtDMSO)$_2$-DAD**: IC$_{50}$ = 43.7 μM). Only **(m-4F-PtDMSO)$_4$DAB(PA)$_4$** showed stronger cytotoxicity and reduced the cell growth in a concentration of 5 μM by 50% after an incubation time of 150 h (see Figure 5).

The inactivity of the mononuclear and the low activity of dinuclear [alkylamine]platinum(II) complexes might be the consequence of inactivation reactions prior to the transport into the cells. Therefore, we performed a modified cell culture experiment on the example of **(m-4F-PtDMSO)$_2$-DAN**. Preincubation of the cells with **(m-4F-PtDMSO)$_2$-DAN** in FCS-free EMEM for 6 h followed by incubation for 150 h under common conditions strongly increased the cytotoxicity (IC$_{50}$ = 12 μM). It is therefore very likely that the strong protein binding as discussed above prevented antiproliferative effects.

Conclusion

In this structure−activity study, we demonstrated that mono-, di-, and tetranuclear [*meso*-1,2-bis(4-fluorophenyl)ethylenediamine][alkylamine]platinum(II) complexes accumulated in tumor cells and bound in high amount to the DNA if inactivation reactions in the cell culture medium can be excluded. The linearity between uptake and complex concentration in the medium corresponds to an accumulation ratio independent from initial concentration of the complex and demonstrates a passive transport across the cell membrane. The most efficient compound was **(m-4F-PtDMSO)$_4$DAB(PA)$_4$**. The DAB(PA)$_4$ polyimine dendrimer seems to operate as a carrier for the shuttling of platinum into the cell nuclei. The kind of DNA binding, however, was not suitable to cause high cytotoxic effects. Therefore, we will modify this concept in such a way that the platinum complexes are bound to the dendrimer allowing a release after the transfer into the cells.

Experimental Section

Synthesis. All reagents and solvents were purchased from ACROS ORGANICS, Fluka Chemie, Lancaster, Merck, or Sigma-Aldrich. ^{1}H NMR: Avance DPX-400 spectrometer (Bruker,

Karlsruhe/Germany) at 400 MHz (internal standard: TMS). Elemental analyses: Microlaboratory of the Free University of Berlin. [*meso*-1,2-Bis(4-fluorophenyl)ethylenediamine][sulfinylbis[methane]-S]sulfatoplatinum(II) **(m-4F-PtDMSO-SO_4)** was prepared as already described.[17]

General Procedure for the Preparation of the [Alkylamine]-platinum(II) Complexes. The solution of the respective amine ligand (monoamine 0.1 mmol, diamine 0.05 mmol, and DAB$(PA)_4$ 0.025 mmol) and [*meso*-1,2-bis(4-fluorophenyl)ethylenediamine]-sulfatoplatinum(II) (0.1 mmol) in 5 mL of DMSO was stirred for 24−36 h with protection from light. Subsequently, the precipitated [alkylamine][sulfinylbis[methane]-S]platinum(II) complexes were separated by suction filtration and dried under vacuum at 50 °C. The transfer into the [alkylamine]chloroplatinum(II) complexes was performed by treatment of an aqueous solution of the respective PtDMSO complex with 3 mL of 0.1 N HCl. The precipitate was sucked off and dried at 50 °C over P_2O_5.

[*meso*-1,2-Bis(4-fluorophenyl)ethylenediamine][sulfinylbis-[methane]-S][propylamine]platinum(II)sulfate (m-4F-PtDMSO-PA). Yield: 37.94 mg (0.056 mmol); 56.11% white powder. ^{1}H NMR (D_2O): δ 0.96 (t, 3H, CH_3), 1.65−1.72 (m, 2H, CH_2), 2.90 (t, 2H, N−CH_2), 3.78 (s, 6H, CH_3), 4.70 (m, 2H, CH−benzyl), 7.02−7.15 (m, 4H, Ar−H), 7.16−7.23 (m, 2H, Ar−H), 7.25−7.32 (m, 2H, Ar−H). Anal. ($C_{19}H_{29}F_2N_3S_2O_5Pt$) C, H, N.

[*meso*-1,2-Bis(4-fluorophenyl)ethylenediamine][sulfinylbis-[methane]-S][butylamine]platinum(II)sulfate (m-4F-PtDMSO-BA). Yield: 33.36 mg (0.048 mmol); 48.34% white powder. ^{1}H NMR (D_2O): δ 0.93 (t, 3H, CH_3), 1.36−1.46 (m, 2H, CH_2), 1.62−1.76 (m, 2H, CH_2), 2.96 (t, 2H, N−CH_2), 3.68 (s, 6H, CH_3), 4.62 (m, 2H, CH−benzyl), 7.04−7.14 (m, 4H, Ar−H), 7.17−7.23 (m, 2H, Ar−H), 7.24−7.31 (m, 2H, Ar−H). Anal. ($C_{20}H_{31}F_2N_3S_2O_5$-Pt) C, H, N.

[*meso*-1,2-Bis(4-fluorophenyl)ethylenediamine][sulfinylbis-[methane]-S][hexylamine]platinum(II)sulfate (m-4F-PtDMSO-HA). Yield: 44.24 mg (0.062 mmol); 61.61% white powder. ^{1}H NMR (D_2O): δ 0.89 (t, 3H, CH_3), 1.29−1.40 (m, 6H, CH_2), 1.68−1.79 (m, 2H, CH_2), 2.95 (t, 2H, N−CH_2), 3.70 (s, 6H, CH_3), 4.60 (m, 2H, CH−benzyl), 7.04−7.13 (m, 4H, Ar−H), 7.15−7.22 (m, 2H, Ar−H), 7.23−7.30 (m, 2H, Ar−H). Anal. ($C_{22}H_{35}F_2N_3S_2O_5$-Pt) C, H, N.

Di(*meso*-1,2-bis(4-fluorophenyl)ethylenediamine)di(sulfinylbis-[methane]-S)(μ-1,6-diaminohexane-N:N′)diplatinum(II)disulfate (m-4F-PtDMSO-DAH). Yield: 33.04 mg (0.021 mmol); 42.73% white powder. ^{1}H NMR (D_2O): δ 1.40−1.48 (m, 4H, CH_2), 1.68−1.79 (m, 4H, CH_2), 2.98 (t, 4H, N−CH_2), 3.71 (s, 12H, CH_3), 4.58−4.69 (m, 4H, CH−benzyl), 7.05−7.13 (m, 8H, Ar−H), 7.18−7.23 (m, 4H, Ar−H), 7.25−7.31 (m, 4H, Ar−H). Anal. ($C_{38}H_{56}F_4N_6S_4O_{10}Pt_2$) C, H, N.

Di(*meso*-1,2-bis(4-fluorophenyl)ethylenediamine)di(sulfinylbis-[methane]-S)(μ-1,9-diaminononane-N:N′)diplatinum(II)disulfate (m-4F-PtDMSO-DAN). Yield: 56.85 mg (0.036 mmol); 71.57% white powder. ^{1}H NMR (D_2O): δ 1.30−1.42 (m, 10H, CH_2), 1.63−1.77 (m, 4H, CH_2), 2.98 (t, 4H, N−CH_2), 3.72 (s, 12H, CH_3), 4.62−4.70 (m, 4H, CH−benzyl), 7.05−7.13 (m, 8H, Ar−H), 7.18−7.23 (m, 4H, Ar−H), 7.25−7.31 (m, 4H, Ar−H). Anal. ($C_{41}H_{62}F_4N_6S_4O_{10}Pt_2$) C, H, N.

Di(*meso*-1,2-bis(4-fluorophenyl)ethylenediamine)di(sulfinyl-bis[methane]-S)(μ-1,12-diaminododecane-N:N′)diplatinum(II)disulfate (m-4F-PtDMSO-DAD). Yield: 50.85 mg (0.031 mmol); 62.37% white powder. ^{1}H NMR (D_2O): δ 1.33−1.46 (m, 12H, CH_2), 1.61−1.70 (m, 8H, CH_2), 2.99 (t, 4H, N−CH_2), 3.48 (s, 12H, CH_3), 4.55−4.67 (m, 4H, CH−benzyl), 6.99−7.09 (m, 8H, Ar−H), 7.17−7.28 (m, 4H, Ar−H), 7.32−7.43 (m, 4H, Ar−H). Anal. ($C_{44}H_{68}F_4N_6S_4O_{10}$-$Pt_2$) C, H, N.

Tetra(*meso*-1,2-bis(4-fluorophenyl)ethylendiamine)di(sulfinyl-bis[methane]-S)(N,N,N′,N′-tetrakis(3-aminopropyl)butane-1,4-diamine)tetraplatinum(II)tetrasulfate (m-4F-PtDMSO)$_4$DAB-(PA)$_4$. Yield: 52.44 mg (0.016 mmol); 63.26% white powder. ^{1}H NMR (D_2O): δ 1.51−1.68 (m, 4H, CH_2), 2.20 (t, 12H, N−CH_2), 2.72−2.88 (m, 8H, CH_2), 2.92−3.05 (t, 8H, N−CH_2), 3.65 (s, 24H, CH_3), 4.56−4.63 (m, 8H, CH−benzyl), 6.91−7.08 (m, 16H, Ar−H), 7.09−7.20 (m, 8H, Ar−H), 7.21−7.35 (m, 8H, Ar−H). Anal. ($C_{80}H_{120}N_{14}F_8S_8O_{20}Pt_4$) C, H, N.

Chloro[*meso*-1,2-bis(4-fluorophenyl)ethylenediamine]-[propylamine]platinum(II)chloride (m-4F-PtCl-PA). m-4F-PtCl-PA was prepared from **m-4F-PtDMSO-PA** (0.1 mmol). Yield: 38.58 mg (0.067 mmol); 67.38% colorless powder. ^{1}H NMR ([D7]-DMF): δ 0.94 (t, 3H, CH_3), 1.73−1.80 (m, 2H, CH_2), 2.82 (t, 2H, N−CH_2), 4.52−4.63 (m, 2H, CH−benzyl), 5.45 (br, 2H, NH_2), 5.58 (br, 1H, NH), 6.41 (br, 2H, NH_2), 7.04−7.11 (m, 4H, Ar−H), 7.12 (br, 1H, NH), 7.50−7.58 (m, 4H, Ar−H). Anal. ($C_{17}H_{23}F_2Cl_2N_3$-Pt) C, H, N.

Chloro[*meso*-1,2-bis(4-fluorophenyl)ethylenediamine][butylamine]platinum(II)chloride (m-4F-PtCl-BA). m-4F-PtCl-BA was prepared from **m-4F-PtDMSO-BA** (0.1 mmol). Yield: 45.50 mg (0.077 mmol); 77.59% colorless powder. ^{1}H NMR ([D7]-DMF): δ 0.84 (t, 3H, CH_3), 1.32−1.40 (m, 2H, CH_2), 1.70−1.78 (m, 2H, CH_2), 2.75 (t, 2H, N−CH_2), 4.52−4.59 (m, 2H, CH−benzyl), 5.32 (br, 2H, NH_2), 5.61 (br, 1H, NH), 6.36 (br, 2H, NH_2), 7.08−7.17 (m, 4H, Ar−H), 7.24 (br, 1H, NH), 7.53−7.59 (m, 4H, Ar−H). Anal. ($C_{18}H_{25}F_2Cl_2N_3Pt$) C, H, N.

Chloro[*meso*-1,2-bis(4-fluorophenyl)ethylenediamine]-[hexylamine]platinum(II)chloride (m-4F-PtCl-HA). m-4F-PtCl-HA was prepared from **m-4F-PtDMSO-HA** (0.1 mmol). Yield: 48.02 mg (0.078 mmol); 78.16% colorless powder. ^{1}H NMR ([D7]-DMF): δ 0.83 (t, 3H, CH_3), 1.25−1.40 (m, 6H, CH_2), 1.70−1.80 (m, 2H, CH_2), 2.80 (t, 2H, N−CH_2), 4.50−4.61 (m, 2H, CH−benzyl), 5.32 (br, 2H, NH_2), 5.60 (br, 1H, NH), 6.31 (br, 2H, NH_2), 7.08−7.19 (m, 4H, Ar−H), 7.24 (br, 1H, NH), 7.51−7.61 (m, 4H, Ar−H). Anal. ($C_{20}H_{29}F_2Cl_2N_3Pt$) C, H, N.

Di(*meso*-1,2-bis(4-fluorophenyl)ethylenediamine)dichloro(μ-1,6-diaminohexane-N:N′)diplatinum(II)dichloride (m-4F-PtCl-DAH). m-4F-PtCl-DAH was prepared from **m-4F-PtDMSO-DAH** (0.1 mmol). Yield: 50.33 mg (0.037 mmol); 37.56% colorless powder. ^{1}H NMR ([D7]-DMF): δ 1.25−1.30 (m, 4H, CH_2), 1.74−1.83 (m, 4H, CH_2), 2.52 (t, 4H, N−CH_2), 4.51−4.59 (m, 4H, CH−benzyl), 5.20 (br, 2H, NH), 5.41 (br, 2H, NH), 5.68 (br, 2H, NH), 6.21 (br, 3H, NH), 6.35 (br, 2H, NH), 7.03−7.15 (m, 8H, Ar−H), 7.24 (br, 1H, NH), 7.48−7.52 (m, 4H, Ar−H), 7.53−7.58 (m, 4H, Ar−H). Anal. ($C_{34}H_{44}F_4Cl_4N_6Pt_2$) C, H, N.

Di(*meso*-1,2-bis(4-fluorophenyl)ethylenediamine)dichloro(μ-1,9-diaminononane-N:N′)diplatinum(II)dichloride (m-4F-PtCl-DAN). m-4F-PtCl-DAN was prepared from **m-4F-PtDMSO-DAN** (0.1 mmol). Yield: 65.79 mg (0.048 mmol); 47.61% colorless powder. ^{1}H NMR ([D7]-DMF): δ 1.25−1.41 (m, 10H, CH_2), 1.70−1.83 (m, 4H, CH_2), 2.95 (t, 4H, N−CH_2), 4.53−4.66 (m, 4H, CH−benzyl), 5.22 (br, 2H, NH), 5.40 (br, 2H, NH), 5.64 (br, 2H, NH), 6.27 (br, 6H, NH), 7.04−7.19 (m, 8H, Ar−H), 7.49−7.60 (m, 8H, Ar−H). Anal. ($C_{37}H_{50}F_4Cl_4N_6Pt_2$) C, H, N.

Di(*meso*-1,2-bis(4-fluorophenyl)ethylenediamine)dichloro(μ-1,12-diaminododecane-N:N′)diplatinum(II)dichloride (m-4F-PtCl-DAD). m-4F-PtCl-DAD was prepared from **m-4F-PtDMSO-DAD** (0.1 mmol). Yield: 48.11 mg (0.034 mmol); 33.78% colorless powder. ^{1}H NMR ([D7]-DMF): δ 1.22−1.45 (m, 12H, CH_2), 1.70−1.85 (m, 8H, CH_2), 2.55 (t, 4H, N−CH_2), 4.51−4.72 (m, 4H, CH−benzyl), 5.30 (br, 4H, NH), 6.19 (br, 8H, NH), 6.99−7.15 (m, 8H, Ar−H), 7.46−7.66 (m, 8H, Ar−H). Anal. ($C_{40}H_{56}F_4Cl_4N_6Pt_2$) C, H, N.

Biological Methods. General. Chemicals were purchased from Sigma and Fluka. Drugs were freshly prepared as stock solution in dimethylformamide (DMF) or H_2O and diluted with cell culture media or buffer when used for the biochemical experiments (final DMF concentration: 0.1% (v/v)). Platinum amounts were determined by graphite furnace atomic absorption spectroscopy (GF-AAS) with deuterium background correction (AAS vario 6; AnalytikJena AG) using a detection wavelength of 265.9 nm. The used program consisted of drying at 105 °C for 30 s (ramp rate = 10 °C/s) and at 120 °C for 20 s (ramp rate = 15 °C/s), ashing at 500 °C for 30 s (ramp rate = 45 °C/s), at 1000 °C for 10 s (ramp rate = 100 °C/s), and at 1700 °C for 10 s (ramp rate = 300 °C/s) as well as atomizing at 2400 °C for 4 s (ramp rate = 1500 °C/s). Purge gas flow-rate was 2 L Argon/min except at the atomization

where it was stopped. Limit of detection was approximately 0.005 mg/L (injection volume: 20 μL).

Stability in Physiological Buffers. The model compound **(m-4F-PtDMSO)$_2$-DAH** (2 mg/mL) was incubated in physiological NaCl solution (0.9% (w/v)), in Delbecco's buffer (137.0 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4) or EMEM at 37 °C. An appropriate aliquot was acidified to pH 2.7 with phosphoric acid and immediately analyzed by capillary electrophoresis (Agilent 3D system equipped with a diode array detector; running buffer: 0.02 M NaH_2PO_4 adjusted to pH 2.7 with H_3PO_4; the applied voltage was 30 kV and the cassette temperature was set to 20 °C). Samples were injected by pressure (50 mbar for 4 s). At the beginning of a sequence, the capillary was flushed with 1 M NaOH for 5 min, twice distilled water and running buffer. Between each run the capillary was flushed with running buffer for 5 min.

Binding Behavior to HSA. A total of 3 μM of each compound was incubated with HSA (40 mg/mL) in Delbecco's buffer. Aliquots were taken after appropriate incubation periods and were 2-fold diluted with ice-cold ethanol. The probe was stored at −18 °C for at least 4 h after vigorous stirring. Then the protein solution was centrifuged (4000*g*, 4 °C, 10 min), and 300 μL of the supernatant was diluted with 100 μL of bidistilled water and stabilized by addition of 100 μL of hydrochloric acid (18% w/v) for GF-AAS determination. The recovery rate was calculated from three independent experiments as the ratio of platinum found in the supernatant and the total amount of platinum. Pseudo-first-order kinetic fitting was done using Origin 7.0 after calculating the found platinum as the percentage of the amount recovered at the beginning of the experiment ($n = 2$).

To determine the recovery by ultrafiltration, aliquots of 200 μL were transferred immediately after addition of the compounds into centrifugal filter devices (Microcon YM-30 Millipore) and were centrifuged (6000*g*, 4 °C, 10 min). A total of 50 μL of the ultrafiltrate was stabilized by addition of 350 μL of Triton-X-100 (1% (w/w)) and 100 μL of hydrochloric acid (18% w/v) for GF-AAS determination. The experiments were performed in triplicate. Calculations were done analogous to the ultrafiltration experiments.

Cell Culture. The human MCF-7 breast cancer cell line was obtained from the American Culture Collection (ATCC, Rockville, MD). The cells were maintained in Eagle's minimal essential medium (EMEM) containing L-glutamine, supplemented with $NaHCO_3$ (2.2 g/L), sodium pyrovate (110 mg/L), gentamycin (50 mg/L), and 10% fetal calf serum (FCS; Gibco Eggenheim, Germany) using 75 cm^2 culture flasks (Nunc) in a water-saturated atmosphere (5% CO_2) at 37 °C. The cells were serially passaged weekly following trypsinization using 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

In Vitro Chemosensitivity Assay. The in vitro testing of the compounds for cytotoxic activity was carried out on exponentially dividing cancer cells according to a previously published microtiter assay.[28] Briefly, in 96-well microtiter assay plates (Nunc), 100 μL of a cell suspension at 7000 cells/mL culture medium was plated into each well and incubated at 37 °C for 3 days in a water-saturated atmosphere (5% CO_2). The desired test concentration was obtained by addition of an adequate volume of a stock solution of the respective compound to the medium. Sixteen wells were used for each test concentration and for the control, which contained the corresponding amount of DMF or H_2O (0.1% v/v), respectively. After appropriate incubation periods, the medium was removed and the cells were fixed with a glutaraldehyde solution and stored at 4 °C. Cell biomass was determined by a crystal violet staining technique. The influence of the complexes on cell growth is expressed as the corrected *T*/*C* value according to the following equations

$$\text{cytostatic effect: } T/C_{\text{corr}}\,[\%] = [(T - C_0)/(C - C_0)] \times 100$$

where *T* (test) and *C* (control) are the optical densities at 590 nm of the crystal violet extract of the cell lawn in the wells (i.e., the chromatin-bound crystal violet extracted with ethanol 70%), C_0 is the density of the cell extract immediately before treatment, and

$$\text{cytocidal effect: } \tau\,[\%] = [(T - C_0)/C_0] \times 100$$

For the automatic determination of the optical density of the crystal violet extract in the wells a microplatereader (Flashscan S19 AnalytikJena AG) was used. The IC_{50} values were determined as previously described[29] with the exception that the incubation time under drug exposure was set to 150 h.

Cellular Uptake Studies. MCF-7 cells were seeded in 6-well plates (Nunc). When the cells reached 50−60% confluency (approximately after 5 days of incubation) the medium was exchanged by serum-free EMEM containing the drugs. The medium was removed after appropriate incubation periods, and the cells were washed with ice-cold PBS. After trypsination, the cells were harvested, washed two times with ice-cold PBS, and centrifuged (2000*g*, 4 °C, 5 min) for storage at −18 °C till analysis. The cell pellet was homogenized by sonification in a Triton X-100 solution (1% (w/w)) and was adequately diluted for protein determination[30] and for the platinum analysis (GF-AAS). Calibrations were done under identical conditions with K_2PtCl_4 standard solutions. The results were calculated as the average of three experiments. The cellular concentration of the compounds in the MCF-7 cells were determined as previously published.[12] The accumulation grade was the ratio of cell-associated platinum and the compound concentration in the medium.

A modified procedure was performed to confirm the validity of Fick's law. Cells were incubated with four different concentrations of the substance. After three or four time intervals, all four concentrations were harvested and treated as described above.

Platinum Content in the Nuclei. MCF-7 cells were seeded in 175 cm^2 flasks and incubated till 60−70% confluency was reached. Then the cells were incubated for 24 h with serum-free medium containing the respective drug. The cells were harvested by trypsination, washed with ice-cold PBS, resuspended in ice-cold RSB buffer (0.01 M Tris-HCl, 0.01 M NaCl, 1.5 mM $MgCl_2$, pH 7.4), and incubated on ice for 5 min. Nonidet-P40 was added to the swollen cells to a concentration of 0.25%, and the cells were lysed by vigorous stirring. After 5 min incubation on ice the raw nuclei were stirred once more and sedimented by centrifugation (1000*g*) at 4 °C for 5 min. The nuclei pellet was resuspended in 0.25 M sucrose containing 3 mM $CaCl_2$ and layered on a 0.88 M sucrose solution. Nuclei were purified by centrifugation for 10 min at 2500*g*. The supernatant was removed, and the nuclei were stored at −18 °C till analysis. For analysis the nuclei were homogenized in 300 μL of Triton X-100 solution (1% (w/w)) by ultrasonification. The homogenate was adequately diluted for protein assay by the method of Bradford[30] and for platinum determination by means of GF-AAS. The results were expressed as the means of three independent experiments as nanograms of platinum per milligrams of nuclear protein.

Platinum Content of Nuclear DNA. MCF-7 cells were treated as described above to yield a raw nuclei pellet, which was incubated with proteinase K and RNAse A in a 0.5% (w/v) sodium dodecylsulfonat containing TE-buffer (10 mmol Tris, 1 mmol EDTA, pH 7.8) for 4 h at 55 °C. Purification was performed by using the chloroform phenol extraction method. DNA pellets were obtained by adding 0.1 aliquots of 3 M sodium acetate solution (pH 5.5) and 2 aliquots of ice-cold ethanol. For GF-AAS analysis this pellet was dissolved in 100 μL of water, and 50 μL of DNA solution was diluted with 50 μL of 0.01 N HCl containing 0.05% (w/w) Triton X-100. For calibration, equal amounts of salmon sperm DNA was added to the K_2PtCl_4 standards. DNA content was measured according to Vytasek in a miniaturized assay.[31] Briefly, the DNA was hydrolyzed by 1 M $HClO_4$. The liberated ribose reacted with 3,5-diaminobenzoic acid under alkaline conditions to a fluorescent chinoline derivative. The reaction was stopped by addition of ice-cold hydrochloric acid (1 M). The fluorescence was measured using a microtiterplate reader (Victor^2 1420 multilabel

counter Perkin-Elmer; filters: Exc. 405 nm; Em. 500 nm). Calibration was done in a range of 0 to 2.5 μg using salmon sperm DNA. The purity of the DNA was determined by UV-spectroscopy. Every working up showed a ratio E_{260}/E_{280} higher than 1.8 and a ratio E_{260}/E_{230} higher than 2.2. The absorption minimum was always lower than 232 nm.[32] Results are expressed as the means of three independent experiments as picograms of platinum per micrograms of nuclear DNA.

Acknowledgment. The technical assistance of M. Baacke is acknowledged. The presented study was supported by Grants Gu285/4-1, Gu285/5-1, and Gu285/5-2 from the Deutsche Forschungsgemeinschaft.

Supporting Information Available: Elemental analysis results of the target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Barnes, K. R.; Lippard, S. J. Cisplatin and related anticancer drugs: Recent advances and insights. In *Metal Ions in Biological Systems*; Sigel, A., Ed.; Marcel Dekker: New York, 2004; Vol. 42 (Metal Complexes in Tumor Diagnosis and as Anticancer Agents), pp 143−177.

(2) Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **2003**, *22*, 7265−7279.

(3) Manzotti, C.; Pratesi, G.; Menta, E.; Di Domenico, R.; Cavalletti, E.; Fiebig, H. H.; Kelland, L. R. M.; Farrell, N.; Polizzi, D.; Supino, R.; Pezzoni, G.; Zunino, F. BBR 3464: A novel triplatinum complex, exhibiting a preclinical profile of antitumor efficacy different from cisplatin. *Clin. Cancer. Res.* **2000**, *6*, 2626−2634.

(4) Kaparkova, J.; Farrell, N.; Brabec, V. Sequence specifity, conformation and recognition by HMG1 protein of major DNA interstrand cross-links of antitumor dinuclear platinum complexes. *J. Biol. Chem.* **2000**, *275*, 15789−15798.

(5) Qu, Y.; Scarsdale, N. J.; Tran, M.-C.; Farrell, N. Cooperative effects in long range 1,4 DNA-DNA interstrand cross-links formed by polynuclear platinum complexes: an unexpected syn orientation of adenine bases outside the binding sites. *J. Biol. Inorg. Chem.* **2003**, *8*, 19−28.

(6) Berners-Price, S. J.; Davies, M. S.; Cox, J. W.; Thomas, D. S.; Farrell, N. Competitive reactions of interstrand and intrastrand DNA-Pt adducts: A dinuclear-platinum complex preferentially forms a 1,4-intrastrand cross-link rather than a 1,2-intrastrand cross-link on binding to a GG 14-mer duplex. *Chem. Eur. J.* **2003**, *9*, 713−725

(7) Di Blasi, P.; Bernareggi, A.; Beggiolin, G.; Piazzoni, L.; Menta, E.; Formento, M. L. Cytotoxicity, cellular uptake and DNA binding of the novel trinuclear platinum complex BBR3464 in sensitive and cisplatin resistant murine leukemia cells. *Anticancer Res.* **1998**, *18*, 3113−3118.

(8) Perego, P.; Gatti, L.; Caserini, C.; Supino, R.; Colangelo, D.; Leone, R.; Spinelli, S.; Farrell, N.; Zunino, F. The cellular basis of the efficacy of the trinuclear platinum complex BBR3464 against cisplatin-resistant cells. *J. Inorg. Biochem.* **1999**, *77*, 59−64.

(9) Jansen, B. A. J.; van der Zwan, J.; Reedijk, J.; den Dulk, H.; Brouwer, J. A tetranuclear platinum compound designed to overcome cisplatin resistance. *Eur. J. Inorg. Chem.* **1999**, 1429−1433.

(10) Müller, R.; Gust, R.; Jennerwein, M.; Reile, H.; Laske, R.; Krischke, W.; Bernhardt, G.; Spruss, T.; Engel, J.; Schönenberger, H. Tumor inhibiting [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) complexes. Part I: Synthesis. *Eur. J. Med. Chem.* **1989**, *24*, 341−348.

(11) Bernhardt, G.; Reile, H.; Spruss, T.; Koch, M.; Gust, R.; Schönenberger, H.; Hollstein, M.; Lux, F.; Engel, J. (±)-(D,L)-[1,2-Bis(4-fluorophenyl)ethylenediamine]dichloroplatinum(II). *Drugs Fut.* **1991**, *16*, 899−903.

(12) Gust, R.; Schnurr, B.; Krauser, R.; Bernhardt, G.; Koch, M.; Schmid, B.; Hummel, E.; Schönenberger, H. Stabilty and cellular studies of [rac-1,2-bis(4-fluorophenyl)ethylenediamine][cyclobutane-1,1-dicarboxylato]platinum(II), a novel, highly active carboplatin derivative. *J. Cancer Res. Clin. Oncol.* **1998**, *124*, 585−597.

(13) Gust, R.; Krauser, R.; Schmid, B.; Schönenberger, H. Breast cancer inhibiting diastereomeric diacetato[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) derivatives − Synthesis and studies on the relationship between reactivity and antitumor activity. *Inorg. Chim. Acta* **1996**, *250*, 203-218.

(14) Lau, J. K.-C.; Deubel, D. V. Quantum chemical studies of metals in medicine. IV. Loss of ammine from platinum(II) complexes: Implications for cisplatin inactivation, storage and resistance. *Chem. Eur. J.* **2005**, *11*, 2849−2855.

(15) Wang, Y.; Farrell, N.; Burgess, J. D. Direct evidence for preassociation preceding covalent binding in the reaction of cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with surface immobilized oligonucleotides. *J. Am. Chem. Soc.* **2001**, *123*, 5576−5577.

(16) Hartinger, C. G.; Timerbaev, A. R.; Keppler, B. K. Capillary electrophoresis in anti-cancer metallodrug research: advances and future challenges. *Electrophoresis* **2003**, *24*, 2023−2037.

(17) Gust, R.; Heinrich, H.; Krauser, R.; Schönenberger, H. [Meso- and rac-1,2-bis(4-fluorophenyl)ethylenediamine]chloro[sulfinyl-bis-(methane)-S]platinum(II) chloride new water soluble platinum complexes with high anti-breast cancer activities. *Inorg. Chim. Acta* **1999**, *285*, 184−189.

(18) Farrell, N.; Kiley, D. M.; Schmidt, W.; Hacker, M. P. Chemical properties and antitumor activity of complexes of platinum containing substituted sulfoxides $[PtCl(R'R''SO)(diamine)]NO_3$. Chirality and leaving-group ability of sulfoxide affecting biological activity. *Inorg. Chem.* **1990**, *29*, 397−403.

(19) Lempers, E. L. M.; Bloemink, M. J.; Reedejk, J. Reactions products of [Pt(ethylenediamine)(dimethylsulfoxide)Cl]Cl and [Pt(ethylenediamine)Cl_2] with d(GpG) and 5′GMP. Unambiguous evidence for stable 1:1 intermediate N7 adducts with coordinated dimethyl sulfoxide. *Inorg. Chem.* **1991**, *30*, 201−206.

(20) Kratz, F. Interactions of antitumor metal complexes with serum proteins. Perspectives for anticancer drug development. In *Metal Complexes in Cancer Chemotherapy*; Keppler, B. K., Ed.; VCH: Weinheim, 1993; pp 391−429.

(21) Trynda-Lemiesz, L.; Kozlowski, H.; Keppler, B. K. Effect of cis-, trans-diamminedichloroplatinum(II) and DBP on human serum albumin. *J. Inorg. Biochem.* **1999**, *77*, 141−146.

(22) Timerbaev, A. R.; Aleksenko, S. S.; Polec-Pawlak, K.; Ruzik, R.; Semenova, O.; Hartinger, C. G.; Oszwaldowski, S.; Galanski, M.; Jarosz, M.; Keppler, B. K. Platinum metallodrug-protein binding studies by capillary electrophoresis-inductively coupled plasma-mass spectrometry: Characterization of interactions between Pt(II) complexes and human serum albumin. *Electrophoresis* **2004**, *25*, 1988−1995.

(23) Shearan, P.; Fernandez Alvarez, J. M.; Smyth, M. R. Adsorptive voltammetric investigation of the interaction of cisplatin with cystine and human serum albumin. *J. Pharm. Biomed. Anal.* **1990**, *8*, 555−561.

(24) Endo, T.; Kimura, O.; Sakata, M. Carrier-mediated uptake of cisplatin by the OK renal epithelial cell line. *Toxicology* **2000**, *146*, 187−195.

(25) Amtmann, E.; Zöller, M.; Wesch, H.; Schilling, G. Antitumoral activity of a sulphur-containing platinum complex with an acidic pH optimum. *Cancer Chemother. Pharmacol.* **2001**, *41*, 461−466.

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

(27) Qu, Y.; Harris, A.; Petz, A.; Kabolizadeh, P.; Penazova, H.; Farrell, N. Synthesis and DNA conformational changes of noncovalent polynuclear platinum complexes. *J. Inorg. Biochem.* **2004**, *98*, 1591−1598.

(28) Reile, H.; Birnböck, H.; Bernhardt, G.; Spruss, T.; Schönenberger, H. Computerized determination of growth kinetic curves and doubling times from cells in microculture. *Anal. Biochem.* **1990**, *187*, 262−267.

(29) Ott, I.; Schmidt, K.; Kircher, B.; Schumacher, P.; Wiglenda, T.; Gust, R. Antitumor-active cobalt-alkyne complexes derived from acetylsalicylic acids studies on the mode of drug action. *J. Med. Chem.* **2005**, *48*, 622−629.

(30) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(31) Vytasek, R. A sensitive fluorometric assay for the determination of DNA. *Anal. Biochem.* **1982**, *120*, 243−248.

(32) Laws, G. M.; Adams, S. P. Measurement of 8-OHdG in DNA by HPLC/ ECD: The importance of DNA purity. *Biotechniques* **1996**, *20*, 36−38.

JM050845R