Amide-Based Prodrugs of Spermidine-Bridged Dinuclear Platinum. Synthesis, DNA Binding, and Biological Activity

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The chemistry and biology of acetyl-protected spermidine-bridged dinuclear platinum complexes [{*trans*-$PtCl(NH_3)_2]_2$-μ-$NH_2(CH_2)_3N(COR)(CH_2)_4NH_2]X_2$ (R = H, X = Cl (1,1/t,t-spermidine, BBR3571); R = CH_3 , X = Cl (**2**); R = CH_2 Cl, X = ClO_4 (**3**); R = CF_3 , X = Cl (**4**)) are compared with their carbamate analogues. The compounds are potential prodrugs for the parent compound **1**, a highly potent antitumor agent. At pH 6–8 hydrolysis of the blocking group with the release of the "parent" protonated species follows the order 4 > 3 ≫ 2. For **4**, rate constants for the deprotection increase in this pH range. The DNA binding profile of **4** is similar to the Boc derivative, confirming the central influence of charge on DNA binding properties. The differences in cytotoxicity for the protected compounds in ovarian carcinoma cell lines sensitive and resistant to cisplatin cannot completely be explained by spontaneous release of 1,1/t,t-spermidine at physiological pH. Inherent cytotoxicity and cell line specificity may contribute to the observed behavior. The properties of the compounds present them also as possible "second-generation" analogues of the clinically relevant trinuclear complex [{*trans*-$PtCl(NH_3)_2$}$_2$-μ-*trans*-$Pt(NH_3)_2(NH_2(CH_2)_6NH_2)_2](NO_3)_4$, (**8**, BBR3464).

Introduction

Polynuclear platinum complexes containing polyamine linkers of variable chain length represent a novel class of antitumor agents with an activity profile distinctly different from profiles of mononuclear platinum drugs such as cisplatin or carboplatin.[1,2] The trinuclear complex [{*trans*-$PtCl(NH_3)_2$}$_2$-μ-*trans*-$Pt(NH_3)_2$-$(NH_2(CH_2)_6NH_2)_2](NO_3)_4$ (BBR3464[1] or 1,0,1/t,t,t, **8**) was the first compound of this class to enter clinical trials. Second-generation analogues of **8** contain polyamine linkers such as spermine and spermidine (Figure 1) in which the electrostatic and hydrogen binding properties of the central platinum unit of the trinuclear complex are replicated by the quaternary nitrogen atoms of the chain. The remarkable potency of these compounds with cytotoxicity in the micromolar to nanomolar range[3] results in a relatively narrow therapeutic index that could represent a problem for the clinical application of these compounds.

In order to overcome these deficiencies, we suggested the possibility of prodrug delivery of a less toxic and better tolerated derivative of the active species.[4] We envisaged Pt polyamine compounds bearing blocking groups on the secondary nitrogen atoms of the linker chain as suitable candidates for this approach. BOC-protected spermidine and spermine complexes of this type are well-known as direct precursors in the synthesis of the free polyamine drugs 1,1/t,t-spermidine and 1,1/t,t-spermine, respectively.[5] These blocked polyamine-bridged complexes are 2–3 orders of magnitude less cytotoxic than the unprotected analogues containing the protonated quaternary nitrogens. Release of the active species can be achieved through spontaneous, pH dependent hydrolysis of the blocking groups. In this manner, a time-dependent slow release of the active drug may be achieved.

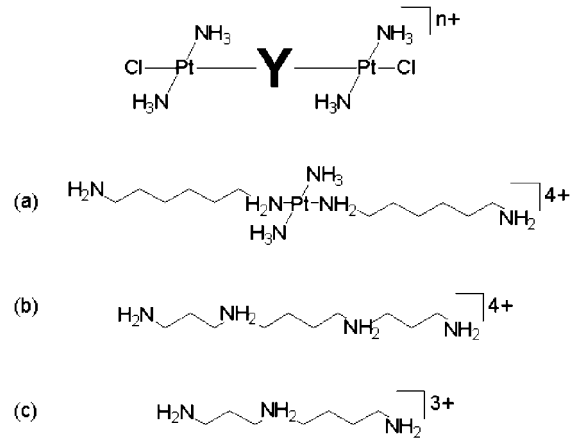

Figure 1. General structure of dinuclear and trinuclear linear Pt polyamine compounds: (a) 1,0,1/t,t,t (**8**); (b) 1,1/t,t-spermine; (c) 1,1/t,t-spermidine (**1**). The abbreviations refer to the number of leaving groups and the configuration of each Pt center; e.g., 1,1/t,t indicates one leaving group trans to the linker chain on each Pt atom of a dinuclear complex.

The rich chemistry of the amino group offers a broad variety of possible protected polyamine complexes that, dependent on the nature of the blocking group, will vary in their susceptibility toward spontaneous or induced cleavage. Carbamate derivatives (BOC *tert*-butyl (**5**), CBz benzyl (**6**), Fmoc fluorenylmethyl (**7**)) of the Pt spermidine complex have been reported previously.[4] The compounds were directly prepared from the unprotected 1,1/t,t-spermidine complex (BBR3571[1]), thereby eliminating the need for selectively protecting and deprotecting the primary amino functions of the spermidine chain, a method used for the original preparation of **5**, 1,1/t,t-N^4-BOC-spermidine.[5] Release of the active 1,1/t,t-spermidine species by spontaneous hydrolysis of **5** and **7** was established by HPLC studies under physiologically relevant pH conditions.[4] Besides carbamates,

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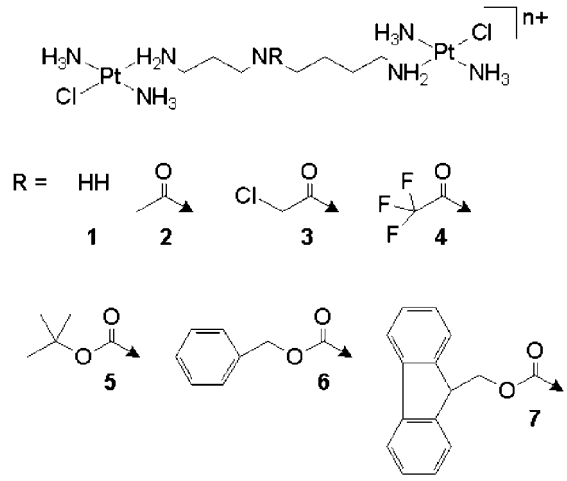

Figure 2. Chemical structures of the dinuclear platinum complexes **1**–**7** (**1**, $n = 3$; **2**–**7**, $n = 2$).

the preparation of amides is the most common approach for the protection of the amino function, and their synthesis and hydrolysis behavior are well described in the literature.[6] Amide derivatives represent a promising area for exploration because a wide range of electronic and steric factors may be systematically modulated. Second, the amide coupling chemistry developed may eventually be exploited to incorporate small peptides with desirable targeting properties such as selective tumor uptake. In this article we report on the incorporation of amide derivatives of 1,1/t,t-spermidine into the prodrug concept. The synthesis and hydrolysis behavior in a relevant pH range is described, and the DNA binding of a representative compound is compared with that of **2**, the Boc derivative, and **8**. Furthermore the in vitro activity of the compounds is evaluated and also compared to compounds of the carbamate series.

Results and Discussion

Chemistry. The structures of the complexes of the amide and carbamate series are illustrated in Figure 2. The synthesis of oxycarbonyl protected (carbamate) spermidine complexes **5**–**7** from 1,1/t,t-spermidine in a water/dioxane mixture at pH 9–11 had been reported earlier.[4] For the amide series this approach did not yield satisfactory results, and a novel synthetic pathway in a water-free solvent was developed. In order to facilitate reactions in organic solvents, compound **1** had to be converted into the acetate derivatives **1a**–**c** (Figure 3), which possess reasonable solubility in methanol, acetonitrile, and water. These intermediates **1a**–**c** were subsequently reacted with the protecting agents (acid anhydrides). Acetic acid anhydride did not require addition of base, while the anhydrides of chloroacetic acid and trifluoroacetic acid gave significantly higher yields in the presence of triethylamine. In the final synthetic step the Pt−Cl bond is restored by reaction with aqueous NaCl.

All compounds were analyzed by HPLC and NMR spectroscopy. The ^{1}H NMR spectra of **1a**–**c** are largely superimposable with the spectrum of **1** except for the presence of signals of the coordinated and anionic acetates in **1a** and **1b**. For compounds **2**–**4**, the signals in the ^{1}H NMR spectra were assigned according to a 2D COSY spectrum of complex **2** (Figure 4). Compared to the precursor compounds, the resonances of the a and a′ protons exhibit a significant downfield shift and are well separated, while for the unprotected compounds only a single multiplet was detected for a/a′. All other signals of the methylene chain experience slight or moderate high field shifts. A splitting found for the c protons in **2**–**4** is indicative of an interaction

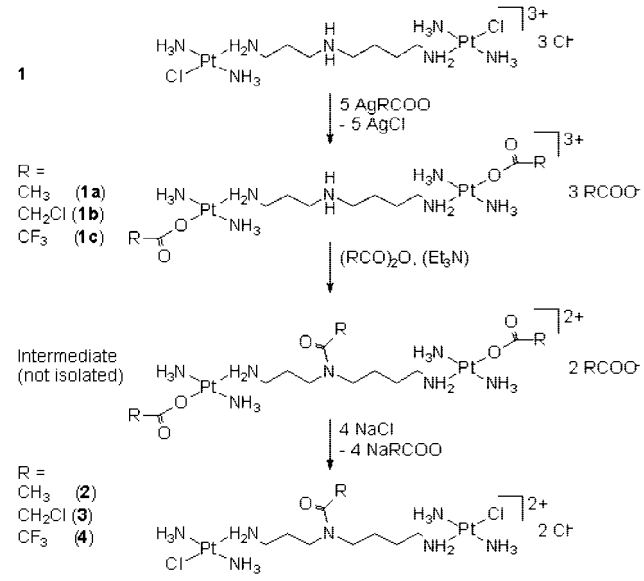

Figure 3. Schematic of the pathway for the synthesis of the acetyl protected platinum spermidine complexes **2**–**4**.

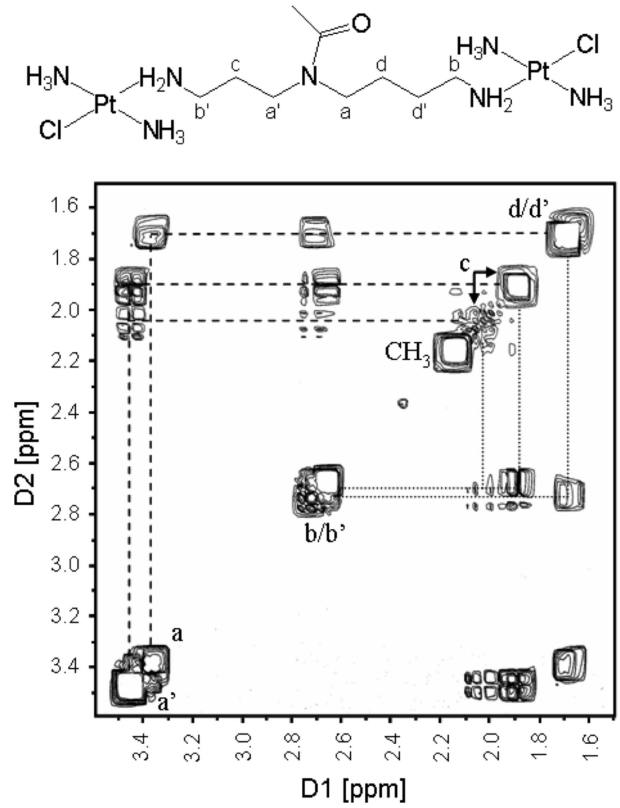

Figure 4. 2D COSY spectrum of compound **2** with assignment of the signals. The coupling of a/a′ and b/b′, respectively, with c and d/d′ is indicated.

between the blocking group and the linker, a feature observed and investigated thoroughly for the Fmoc-protected spermidine complex.[4] A pair of ^{195}Pt spectra recorded for the trifluoroacetyl compounds **1c** and **4** confirmed the changes in the coordination sphere of the Pt centers: In **1c**, the ^{195}Pt shift of −2132 ppm is indicative of the substitution of the "softer" Lewis base chloride in 1,1/t,t-spermidine (δ −2434 ppm) by trifluoroacetate in a PtN_3O coordination sphere, while in **4** (δ −2415 ppm) the original PtN_3Cl coordination sphere is restored.

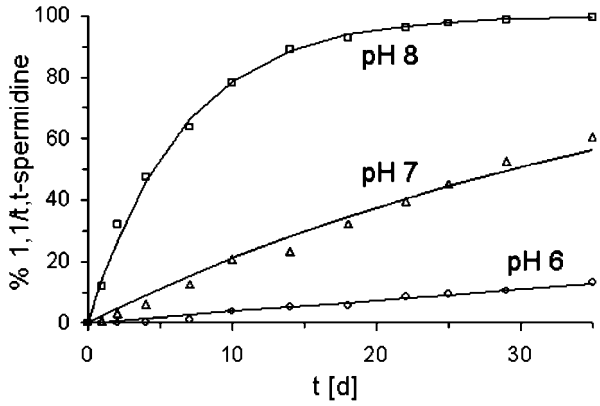

Figure 5. Percentage of 1,1/t,t-spermidine (**1**) found in the HPLC chromatograms of compound **4** at pH 6–8 over a time course of 35 days. The lines show the Scientist fit for a first-order reaction.

Table 1. Rate Constants [s^{-1}] for the Deprotection of N4 Blocked Platinum Spermidine Complexes

pH	4	5	7
8	$(1.79 \pm 0.03) \times 10^{-6}$		$(1.44 \pm 0.07) \times 10^{-7}$
7	$(2.73 \pm 0.06) \times 10^{-7}$	$(3.12 \pm 0.04) \times 10^{-8}$	$(6.12 \pm 0.09) \times 10^{-8}$
6.6		$(3.51 \pm 0.05) \times 10^{-8}$	
6	$(4.44 \pm 0.11) \times 10^{-8}$	$(4.29 \pm 0.05) \times 10^{-8}$	$(9.74 \pm 0.88) \times 10^{-9}$
5		$(4.65 \pm 0.04) \times 10^{-8}$	$(3.99 \pm 0.96) \times 10^{-9}$

Hydrolysis Studies. The hydrolysis of the blocking groups on the N4 position of compounds **2**–**4** was monitored at 37 °C over a pH range of 6–8. By the start of the reaction the HPLC chromatograms of all samples displayed only signals of the protected species, together with minor impurities (<3% of the overall integration in all cases), but no 1,1/t,t-spermidine, **1**. The HPLC chromatograms of **2**, the acetyl protected spermidine complex, did not show any changes over a time period of 35 days, indicating the excellent stability of the acetyl group in the observed pH range.

Trifluoroacetyls are generally cleaved under mild conditions,[11] and the HPLC profile of compound **4** shows indeed the conversion of the N4 blocked species into the unprotected, protonated form, which is clearly separated in the chromatograms because of its increased cationic charge. The amount of **1** released over time is depicted in Figure 5. No other products were detected; trifluoroacetate, originating from the hydrolysis reaction, is not retained on the column under the present conditions and coelutes with the other anions (Cl^- from **2**, NO_3^- from pH adjustment). In order to obtain rate constants for the conversion of **4** to **1**, the integral values of the chromatograms were corrected for the different absorption of the species at the observed wavelength (ϵ_{215} ($L \cdot mol^{-1} \cdot cm^{-1}$): **1**, 3.64×10^3; **2**, 7.62×10^3; **3**, 10.4×10^3; **4**, 9.46×10^3; for details see ref 4). Concentrations were calculated on the basis of the assumption that the sum of the concentrations of both species at every time point equals the original concentration of **4** at the start of the experiment. First-order rate constants were obtained using the program MicroMath Scientist, version 2.01. The results are summarized in Table 1 and compared with values obtained for BOC and Fmoc protected spermidine complexes. For better comparison, all rate constants discussed in this article were calculated with the Scientist program; therefore, the values for the BOC and Fmoc complexes in Table 1 vary slightly from the rate constants reported previously,[4] which were obtained graphically. The resulting differences are mostly negligible and do not affect the conclusions we have drawn from them.

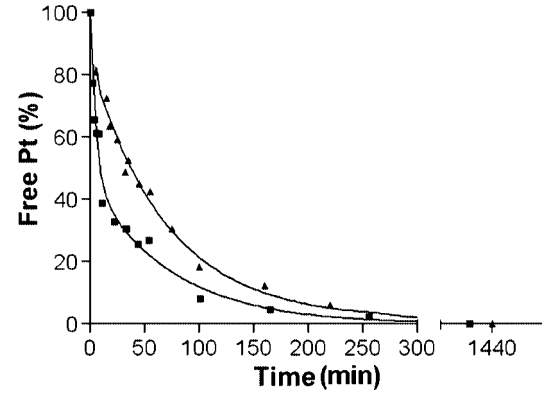

Figure 6. DNA binding of **1** (■) and **4** (▲) as a function of time.

At neutral pH the hydrolysis rates for the protected spermidines of the amide and carbamate series follow the order **4** > **7** > **5** ≫ **2** ∼ **6** (for **3**, see below). Decrease in pH by 1 unit leads to a reduction of the rate constants for **4** and **7** by almost 1 order of magnitude in the observed range, while **5** actually shows a moderate increase in its hydrolysis rate with lower pH. At pH 6, the rate constants follow the order **4** ∼ **5** > **7**. Many solid tumors are known to accumulate lactic acid, resulting in a reduced intracellular pH value.[7] Therefore, the prodrugs most suited for targeting those tumor cells might be the ones that would show increased release of the active species under slightly acidic conditions.

No attempts were made to calculate rate constants for **3** because the hydrolysis behavior of this compound appears to be more complex. The chloroacetate group was chosen because its electronic properties in terms of an oxygen donor ligand should be between those of acetate and trifluoroacetate. Formation of the free spermidine compound **1** is observed within 1 day in the chromatograms, but instead of steadily increasing, the concentration of the active species reaches a plateau within a few days (pH 8, 7–9%; pH 7, 1–2%; pH 6, 4–6%). At the same time several new unidentified species emerge with at least three additional signals detected at longer retention times. The new signals increase steadily over time, while the concentration of **3** constantly decreases. In contrast to the other protection groups of the series, the CH_2Cl residue of the chloroacetyl group is susceptible to nucleophilic attack, a fact frequently used for the removal of the group under mild conditions with reagent such as *o*-phenylenediamine[8] or thioureas.[9,10] Cleavage of the amide bond in **3** leads to the liberation of the free amine **1** and chloroacetate, and all of these species could be involved in side reactions. The situation is further complicated by the fact that chloroacetate is around 2–3 orders of magnitude more basic than trifluoroacetate[11] and is therefore more likely to undergo substitution reaction on the platinum centers, either by direct attack or, more likely, following the spontaneous hydrolysis of the Pt−Cl bond.

DNA Binding Properties. Our previous studies have shown that the presence of the central charge in both trinuclear and spermidine-bridged dinuclear compounds affected both the rate of DNA binding and the amount of interstrand cross-linking.[12,13] It was therefore of interest to examine the DNA binding properties of **4** and compare them with those of the parent **1** (spermidine compound) as well as **5**, the previously studied Boc derivative. The amount of the platinum compounds **1** and **4** bound to calf thymus (CT) DNA increased with time (Figure 6), and after approximately 4 h the two compounds were quantitatively bound. In these binding reactions, the times at

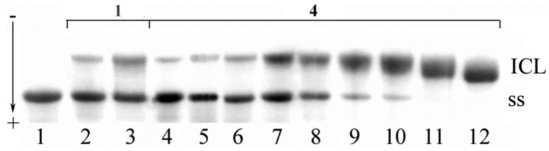

Figure 7. Formation of interstrand CLs by complexes **1** and **4** in linearized pSP73 plasmid (2464 base pairs). Shown is an autoradiogram of denaturing 1% agarose gels of linearized DNA that was 3′-end labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). Information about the lanes are as follows: (1) control, nonplatinated DNA; (2, 3) DNA modified by **1**; (4–12) DNA modified by **4**; (2) $r_b = 1 \times 10^{-4}$; (3) $r_b = 3 \times 10^{-4}$; (4) $r_b = 2.1 \times 10^{-5}$; (5) $r_b = 4.3 \times 10^{-5}$; (6) $r_b = 6.3 \times 10^{-5}$; (7) $r_b = 1.3 \times 10^{-4}$; (8) $r_b = 2.1 \times 10^{-4}$; (9) $r_b = 4.3 \times 10^{-4}$; (10) $r_b = 6.4 \times 10^{-4}$; (11) $r_b = 1.3 \times 10^{-3}$; (12) $r_b = 2.1 \times 10^{-3}$.

which the binding reached 50% ($t_{50\%}$) were 9 and 35 min for **1** and **4**, respectively.

Interstrand Cross-Linking. The frequency of interstrand CLs (% ICL/Pt) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size (r_b is defined as the amount of platinum atoms bound per one nucleotide in DNA). The DNA interstrand cross-linking efficiency of **1** and **4** was almost independent of r_b and was 33% and 90%, respectively (Figure 7). Thus, the DNA interstrand cross-linking efficiency of **1** was significantly higher than that of cisplatin (6%) and similar to that of the trinuclear bifunctional complex **8**. In contrast, **4** showed very high interstrand cross-linking efficiency (~90%).

Sequence Preference of DNA Adducts. In vitro DNA synthesis on double-stranded templates containing the Pt-DNA adducts generated a population of DNA fragments, indicating that these adducts terminate duplex synthesis (Figure 8A, lane 3 (**1**) and lane 4 (**4**)). Sequence analysis of the termination sites produced by **1** and **4** suggests a sequence preference for dG sites and some preference also for dA and dC sites in double-helical DNA (Figure 8B). Both complexes exhibit sequence dependence of the inhibition similar to transplatin. Thus, the new compounds form more blocks on DNA for DNA polymerase than cisplatin, suggesting less regular binding in comparison with cisplatin. Interestingly, the site-specific 1,2-intrastrand CL of **1** (formed on a 30-mer template and containing one adduct only) is a more efficient inhibitor of DNA polymerization than the analogous cisplatin adduct.[14]

The combined DNA binding results may be compared with previous work.[12,13] The presence of the $COCF_3$ group reduces the charge and hydrogen-bond donating capacity of the parent compound **1** (Table 2). The $t_{50\%}$ of **4** at 35 min is very similar to that of the Boc derivative **5** (39 min) as is the frequency of interstrand cross-linking (74% and 90%, respectively). In contrast the present studies confirm the more rapid binding of **1** (9 min) and its relatively lower frequency of interstrand cross-linking of 33%. The results are in agreement with the general trend: positive charge in central linker enhances rate of DNA binding (cf. the 1,1/t,t compound with the simple hexanediamine linker) but concomitantly appears to decrease the frequency of cross-linking; the 1,1/t,t compound has 70–90% interstrand CLs, whereas either **1** or **8** or both are in the 20–40% range (Table 2).

Biological Activity. The in vitro cytotoxicity of the blocked derivatives is given in Table 3. The panel of cell lines chosen included both cisplatin-sensitive and cisplatin-resistant cells so that a variety of phenotypes (based on the mechanisms of resistance) could be examined. Blocking produces significantly less toxic derivatives than the "parent" **1**. The results again confirm the importance of charge in dictating the cytotoxicity and antitumor activity of this series. The reduced cytotoxicity could reflect reduced cellular uptake; protection of the central NH in **1** by the Boc group (**5**) results in decreased cellular uptake.[15] The differences in cytotoxicity in the series of protected compounds cannot completely be explained by the assumption that the cytotoxic properties arise exclusively from the spontaneous release of 1,1/t,t-spermidine at physiological pH. Inherent cytotoxicity and cell line specificity of the protected polyamine complexes are likely factors that contribute to the observed behavior. It is also possible that the different DNA binding properties as described above may be a factor. Whereas the concept of hydrolysis to produce an active species could be a dominant factor in vivo, the in vitro studies done here may reflect a contribution from the "intact" species such as **4** and **5**. The cytotoxicity of platinum compounds correlates well with the doses required to produce in vivo antitumor activity. Whether the approach described here results in enhanced therapeutic indices must await animal studies.

Conclusions

This paper gives a further example of the "prodrug" concept for dinuclear platinum complexes. The results broadly confirm a correlation between time-dependent hydrolysis and cytotoxicity; the trifluoroacetate derivative is more potent than the acetate analogue. The results further confirm that the presence of a central charge is an important factor in the potency of dinuclear and trinuclear platinum complexes. Within the two prodrug series, compounds **4** and **5** appear to have the most promising properties for in vivo evaluation. Their micromolar cytotoxicity suggests that their rate of hydrolysis is suitable for a useful prodrug. Both compounds also display reduced affinity for human serum albumin in comparison to **1** or **8**, suggesting that the rate of hydrolysis may be sufficient to bypass or diminish potentially deactivating reactions with plasma proteins.[26] It was not the purpose of these studies to predict the toxicity of these compounds in animals, but trifluoroacetate at micromolar concentrations is unlikely to exert undue toxic effects. The DNA binding profiles of both derivatives are also similar to the "parent" 1,1/t,t compound with known antitumor activity.[1,2]

An alternative to activation through simple hydrolysis is afforded by enzymatic activation. Various approaches for tumor cell specific enzymatic activation of prodrugs have been developed using antibodies[16,17] or viruses[18,19] to target specific enzymes to tumor cells. The chemistry developed here, especially the synthesis of an N−COR moiety, could have broad applications by choice of R; short linkers can be made with a terminal −COOH or $-NH_2$ capable of conjugation with a host of carrier molecules including peptides, affinity tags, and fluorescent labels. Thus, the chemistry developed here has broad applications to platinum drug delivery. The carboxylate compounds **1a−c** may represent models for incorporation of dinuclear and trinuclear platinum agents into carboxylate or polyglutamate-based polymers and nanoparticle carriers. Further, while the spermidine ("3-4"where 3 and 4 represent the number of carbon atoms) linker has been described here as proof of principle, the chemistry is also applicable to spermine-like linkers. In this case it is noteworthy that the complex [{*trans*-$PtCl(NH_3)_2$}$_2$-μ-($H_2N(CH_2)_6NH(CH_2)_2NH(CH_2)_6NH_2$)]$(NO_3)_4$ (BBR3610, containing a "6-2-6" polyamine linker) is an exceptionally potent agent with nanomolar cytotoxicity.[1,27,28] The entity was developed by systematic design of dinuclear

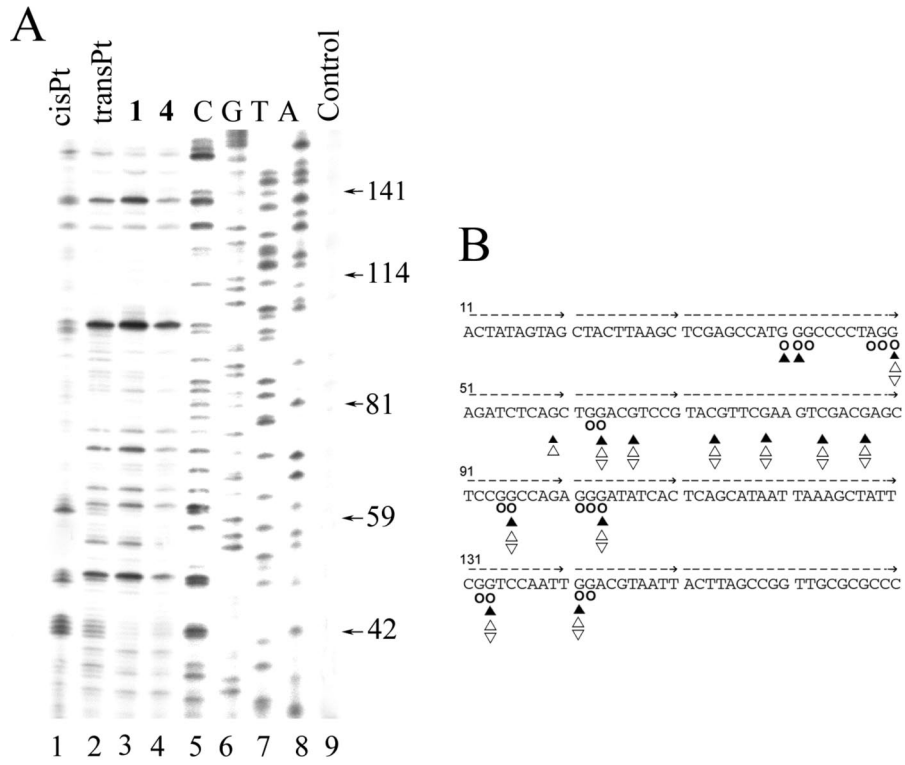

Figure 8. (A) Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by TaKaRa Taq DNA polymerase on the pSP73 plasmid DNA linearized by *Hpa*I restriction enzyme and subsequently modified by platinum complexes. The gel contained the linear amplification products of control, nonplatinated DNA, and DNA treated with the compounds **1** and **4**, cisplatin, or transplatin at $r_b = 0.005$. Information about the lanes are as follows: (1) DNA modified by cisplatin at $r_b = 0.005$; (2) DNA modified by transplatin at $r_b = 0.005$; (3) DNA modified by **1** at $r_b = 0.005$; (4) DNA modified by **4** at $r_b = 0.005$; (5–8) chain-terminated marker DNAs (note that these dideoxy sequencing lanes give the sequence complementary to the template strand); (9) control, nonmodified template. The numbers correspond to the nucleotide sequence numbering of panel B. (B) Schematic diagram showing a portion of the sequence used to monitor inhibition of DNA synthesis on the template containing adducts of the compounds **1** and **4**, transplatin, or cisplatin. The arrows indicate the direction of the synthesis: (open circles) major stop signals from panel A, lane cisPt; (solid triangles) major stop signals from panel A, lane transPt; (open triangles) major stop signals from panel A, lane **1**; (reversed open triangles) major stop signals from panel A, lane **4**. The numbering of the nucleotides in this scheme corresponds to the numbering of the nucleotides in the pSP73 nucleotide sequence map.

Table 2. Effect of Blocking Group on DNA Binding Properties of Spermidine-Linked Dinuclear Compounds[a]

property	1,1/t,t ($n = 6$)	(**1**) 1,1/t,t-spermidine	(**5**) 1,1/t,t-BOC-spermidine	(**4**) 1,1/t,t-$COCF_3$-spermidine	(**8**) 1,0,1/t,t,t
total charge	2+	3+	2+	2+	4+
DNA binding, $t_{1/2}$ (min)	40–60	9.0 (8.2)	9.2	35.0	40
% ICL/adduct at $r_b = 2 \times 10^{-4}$	70–90	33 (40)	74	90	20
refs	13	this work and ref 12	12	this work	13

[a] Average of three experiments.

Table 3. IC_{50} [μM] Evaluation in an Ovarian Carcinoma Cell Line Panel Sensitive and Resistant to Cisplatin

complex	A2780	A2780/CDDP (RF)[a]	CH1	CH1/CDDP (RF)[a]	41M	41M/CDDP (RF)[a]
cisplatin	1.6	12.0 (7.5)	0.34	1.1 (3.2)	2.3	
8	<0.25	<0.25	0.42	0.34 (0.8)	0.20	
1	<0.25	<0.25	0.43	0.35 (0.8)	<0.25	
2	14.0	54.0 (3.9)	7.4	12.5 (1.7)	17.5	24.0 (1.4)
4	6.8	46.0 (6.8)	2.4	5.0 (2.1)	3.7	19.0 (5.1)
5[b]	2.1	19.0 (9.0)	8.0	11.0 (1.4)	17.0	
6[b]	24.0	100.0 (4.2)	25.0	43.0 (1.7)	>100	
7[b]	0.84	65.0 (77)	46.0	62.0 (1.3)	>100	

[a] Resistance factor in parentheses. RF = (IC_{50} resistant)/(IC_{50} parent line). [b] Values for A2780 and CH1 from ref 4.

polyamine-linked compounds to produce similar distances between platinating Pt−Cl units as the trinuclear **8**.[1] Most recently, carboxylate derivatives of 3610 have been described as possible candidates for phase I clinical trials.[29]

Experimental Section

Starting Materials. [{*trans*-$Pt(NH_3)_2Cl]_2$-μ-spermidine-N^1,N^8]-Cl_3 (**1**),[5] [{*trans*-$Pt(NH_3)_2Cl]_2$-μ-N^4-BOC-spermidine-N^1,N^8]Cl_2 (**5**), [{*trans*-$Pt(NH_3)_2Cl]_2$-μ-N^4-CBz-spermidine-N^1,N^8]Cl_2 (**6**), and [{*trans*-$Pt(NH_3)_2Cl]_2$-μ-N^4-Fmoc-spermidine-N^1,N^8]Cl_2 (**7**) were prepared according to the published methods.[4] Silver acetate, silver trifluoroacetate, acetic anhydride, and chloroacetic anhydride were purchased from Aldrich. Trifluoroacetic anhydride was purchased from Fluka. Silver chloroacetate was obtained by dissolving Ag_2O in an aqueous solution of chloroacetic acid (Aldrich). CT DNA (42% G + C, mean molecular mass of about 20 000 kDa) was prepared and characterized as described previously.[20] Plasmids pSP73 (2464 base pairs) were isolated according to standard

procedures. *Eco*RI and *Nde*I restriction endonucleases, thermal cycle dideoxy DNA sequencing kit with $Vent_R(exo^+)$ DNA polymerases, and T4 polynucleotide kinase were purchased from New England Biolabs. Klenow fragment of DNA polymerase I was from Boehringer-Mannheim Biochemica. A primer 5′-d(GATTTAGGT-GACACTATAG) was from BioVendor (Brno, Czech Republic). Acrylamide, bis(acrylamide), and agarose were from Merck KgaA.

Instrumentation. ^{1}H NMR spectra were measured in D_2O solution on a Varian Mercury 300 MHz spectrometer using sodium (trimethylsilyl)propionsulfonate (TSP, $\delta = 0.00$ ppm relative to TMS) as internal reference. ^{195}Pt spectra were recorded in D_2O at 64 MHz using $K_2[PtCl_6]$ as external reference. pH measurements were taken on a Corning 340 pH meter with combined glass electrode. Extinction coefficients were determined with a Jasco V-550 UV/vis spectrophotometer using 1 cm cuvettes. IR spectra were measured as KBr pellets on a Nicolet Nexus 670 FT-IR instrument. Elemental analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ.

Product analysis and hydrolysis studies were carried out on an analytical Beckman System Gold Nouveau HPLC instrument with UV detection at 215 nm. A Synergi Hydro C18 end-capped column (10 μm particle size, dimensions 4 mm × 250 mm) was used with a water/methanol gradient elution method (solvent A, 3% methanol, 0.05 M $NaClO_4$, 1% NaCl; solvent B, 30% methanol, 0.05 M $NaClO_4$, 2% NaCl).

Hydrolysis Study. For the hydrolysis experiments 10^{-3} mmol complex were dissolved in 1 mL of nanopure water. The pH of the solutions was adjusted by addition of HNO_3 (0.1, 0.01 M) and NaOH (0.1, 0.01 M), respectively. The samples were incubated in a water bath at 37 °C for a time course of 35 days. During this period, aliquots of 20 μL were taken from the bulk solution for HPLC analysis. The pH values of the samples were controlled at regular intervals and readjusted if necessary.

[{*trans*-Pt(NH$_3$)$_2$X}$_2$-μ-spermidine-N^1,N^8]X$_3$ (X = CH$_3$COO$^-$ (1a), CH$_2$ClCOO$^-$ (1b), CF$_3$COO$^-$ (1c)). An amount of 1.0 mmol of **1** was dissolved in 50 mL of water, and a total of 4.97 mmol of AgX was added with stirring. Stirring was continued for 24 h at 40 °C in the dark. The mixture was then allowed to come to room temperature and was filtered through Celite. The filtrate was evaporated to dryness, giving a gray residue of **1b** and **1c**, which was redissolved in a minimum amount of water, filtered, and brought to dryness. **1a** was obtained as an oil, which crystallized upon stirring in 40 mL of acetone/diethylether (1:1). The yields for compounds **1a**−**c** were quantitative.

1a. Anal. Calcd for $C_{17}H_{47}N_7O_{10}Pt_2 \cdot 2H_2O$: C, 21.82; H, 5.49; N, 10.48. Found: C, 21.86; H, 5.16; N, 10.57. ^{1}H NMR: δ 1.75 (m, 4 H), 1.96/1.98 (s each, 15 H), 2.11 (m, 2 H), 2.70 (m, 4 H), 3.10 (m, 4H).

1b. Anal. Calcd for $C_{17}H_{42}N_7O_{10}Cl_5Pt_2$: C, 19.05; H, 3.95; N, 9.15; Cl, 16.54. Found: C, 18.93; H, 3.78; N, 8.85; Cl, 16.41. ^{1}H NMR: δ 1.76 (m, 4 H), 2.10 (m, 2 H), 2.71 (m, 4 H), 3.11 (m, 4H), 4.06 (s, 6 H), 4.14 (s, 4 H).

1c. Anal. Calcd for $C_{17}H_{32}N_7O_{10}F_{15}Pt_2 \cdot H_2O$: C, 17.19; H, 2.89; N, 8.26. Found: C, 17.17; H, 2.71; N, 8.17. ^{1}H NMR: δ 1.76 (m, 4 H), 2.10 (m, 2 H), 2.70 (m, 4 H), 3.09 (m, 4H). ^{195}Pt NMR: δ −2132 ppm.

[{*trans*-Pt(NH$_3$)$_2$Cl}$_2$-μ-N^4-CH$_3$CO-Spermidine-N^1,N^8]Cl$_2$ (2). Samples of 0.5 mmol of **1a** and 4.4 mmol of acetic anhydride were combined in 10 mL of methanol and stirred for 2 h at ambient temperature. A total of 30 mL water was added, and the solution was washed with 3 × 30 mL of diethyl ether. The aqueous solution was then brought to dryness, and the remaining oil was dissolved in 10 mL of water. Then an amount of 2.0 mmol of NaCl was added and the pH of the solution was adjusted to 3.8 with 0.5 M HCl. The mixture was stirred for 4 h at room temperature and evaporated to dryness. The residue was recrystallized from methanol and subsequently from ethanol/water (4:1), the final yield being 54%. Anal. Calcd for $C_9H_{33}N_7OCl_4Pt_2$: C, 13.73; H, 4.22; N, 12.45; Cl, 18.01. Found: C, 13.70; H, 4.01; N, 12.17; Cl, 17.74. ^{1}H NMR: δ 1.72 (m, 4 H), 2.16 (s, 3 H), 1.92/2.05 (m each, 2 H), 2.74 (m, 4 H), 3.40 (m, 2 H), 3.47 (m, 2 H). IR: ν_{CO} 1611 cm^{-1}.

[{*trans*-Pt(NH$_3$)$_2$Cl}$_2$-μ-N^4-CH$_2$ClCO-Spermidine-N^1,N^8]-(ClO$_4$)$_2$ (3). An amount of 0.5 mmol of **1b** was suspended in 50 mL of acetonirile, and 1.0 mmol of triethylamine was added with stirring. The suspension was stirred at 40 °C, and a total of 20 mmol of chloroacetic anhydride was added in several portions. After 24 h all undissolved solid is filtered off (unprotected starting compound according to ^{1}H NMR, about 100 mg) and the filtrate was concentrated to dryness. The residue was dissolved in 25 mL of water and washed with 2 × 20 mL of diethyl ether. An amount of 2.5 mmol of NaCl was added, and the aqueous solution was stirred at ambient temperature for 2 h. The solvent was removed in vacuum, and the residue was redissolved in 60 mL of methanol. Undissolved solid was filtered off, and the filtrate was concentrated to a small volume (<5 mL). Addition of 40 mL of acetone/diethyl ether (1:1) caused the product to precipitate. Several recrystallizations from water, water/ethanol, and water/DMF did not yield a pure product (purity of <90% by HPLC). The product was HPLC purified with a semipreparative column (Waters Bondapak C18, 7.8 mm × 300 mm), using a gradient elution method (solvent A, H_2O, 0.025 M $NaClO_4$; solvent B, H_2O, methanol (70:30), 0.025 M $NaClO_4$). The product was obtained as a perchlorate salt and was 99% pure by HPLC, with the final yield being 29%. Anal. Calcd for $C_9H_{32}N_7O_9Cl_5Pt_2$: C, 11.38; H, 3.40; N, 10.32. Found: C, 11.55; H, 3.08; N, 10.29. ^{1}H NMR: δ 1.74 (m, 4 H), 4.41/4.38 (s each, 2 H), 1.93/2.09 (m each, 2 H), 2.73 (m, 4 H), 3.44 (m, 2 H), 3.54 (m, 2 H). IR: ν_{CO} 1642 cm^{-1}.

[{*trans*-Pt(NH$_3$)$_2$Cl}$_2$-μ-N^4-CF$_3$CO-Spermidine-N^1,N^8]Cl$_2$ (4). A total of 0.5 mmol of **1c** was suspended in 40 mL of acetonirile, and 1.0 mmol of triethylamine was added to reach a clear solution. An amount of 30 mmol of trifluoroacetic anhydride was added in several portions, and the mixture was stirred for 24 h at 40 °C. The solvent was removed in vacuum, and 20 mL of acetone and 80 mL of diethyl ether were added to the remaining oil. A white solid was filtered and washed with diethyl ether. The solid was dissolved in 20 mL of water and stirred with 2.5 mmol of NaCl for 2 h (pH 2.4 with 0.5 M HCl). The solution was brought to dryness, and the remaining residue is stirred in 200 mL of methanol. The solution was filtered from some undissolved solid and evaporated in vacuum. The product was recrystallized from water, and the yield was 61%. Anal. Calcd for $C_9H_{30}N_7OCl_4F_3Pt_2$: C, 12.85; H, 3.59; N, 11.65; Cl, 16.86. Found: C, 12.68; H, 3.46; N, 11.20; Cl, 17.08. ^{1}H NMR: δ 1.74 (m, 4 H), 2.01/2.10 (m each, 2 H), 2.73 (m, 4 H), 3.53 (m, 2 H), 3.59 (m, 2 H). ^{195}Pt NMR: δ −2415 ppm. IR: ν_{CO} 1684 cm^{-1}.

DNA Binding. Solutions of double-helical CT DNA at a concentration of 0.032 mg/mL were incubated with **1** and **4** at an r_i of 0.05 in 10 mM $NaClO_4$ at 37 °C (r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA). At various time intervals an aliquot of the reaction mixture was withdrawn and assayed by differential pulse polarography.[21] Interstrand cross-linking efficiency was evaluated in linearized pSP73 plasmid (2464 base pairs). This plasmid DNA was linearized by *Eco*RI (*Eco*RI cuts only once within the pSP73 plasmid), 3′-end-labeled by [α-^{32} P]ATP and modified by the platinum complexes for 24 h at 37 °C in the dark to reach various r_b values. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions. Upon electrophoresis, the 3′-end-labeled strands of linearized plasmid DNA containing no interstrand CLs migrate as a 2464-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species (Figure 7). The intensity of the more slowly migrating band increased with the growing level of the modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition.

Sequence Preference of DNA Adducts. This procedure involved the extension by TaKaRa Taq DNA polymerase (which exhibits the extreme termostability) at the 3′ end of the 5′ end-radioactively labeled primer up to the metal adduct on the template strand. The fragment of pSP73 DNA linearized by *Nde*I (*Nde*I cuts only once

within this plasmid) was obtained as previously described.[22] After deproteinization by phenol/chloroform the modification of this fragment by **1** or **4** was carried out in 10 mM $NaClO_4$ for 48 h at 37 °C to obtain $r_b = 0.005$. CircumVent thermal cycle dideoxy DNA sequencing kit with $Vent_R(exo^+)$ DNA polymerase was used along with the protocol for thermal cycle DNA sequencing with 5′ end-labeled primer recommended by the manufacturer with small modifications.[23] The products of the linear amplification were then examined on DNA sequencing gels, and the sequence specificity of the platinum adduct formation was determined to the exact base pair.

Biological Assays. Cell Culture. A2780, A2780/CDDP, CH1, CH1/CDDP cell lines were used in this study and maintained according to published procedures.[24,25] The IC_{50} values were calculated similar to that described previously.[4] In brief, cells were cultured in RPMI (Gibco, Carlsbad, CA), 5% FBS, 5% BCS, 0.25% penicillin/streptomycin. Cells were routinely passaged at 80–90% confluency. For the MTT assay, cells were plated at a density of 9000 cells per well in a 96-well plate and incubated overnight at 37 °C in a 5% CO_2 atmosphere. Cells were treated with varying drug concentrations for 96 h. After drug removal, cells were washed twice with cold PBS and incubated with MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) (2 mg/mL in PBS). The excess MTT (Sigma; St. Louis, MO) was then removed. The dye was dissolved in 100 μL of DMSO (Sigma; St. Louis, MO), and absorbance was read at 540 nm.

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References

(1) (a) Farrell, N. In *Platinum-Based Drugs in Cancer Therapy*; Kelland, L. R., Farrell, N., Eds.; Humana Press: Totowa, NJ, 2000; pp 321–338. (b) Farrell, N.; Spinelli, S. In *Uses of Inorganic Chemistry in Medicine*; Farrell, N., Ed.; Royal Society of Chemistry: Cambridge, U.K., 1999; pp 124–134.

(2) Farrell, N.; Qu, Y.; Bierbach, U.; Valsecchi, M.; Menta, E. In *30 Years of Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug*; Lippert, B., Ed.; Verlag: Berlin, 1999; pp 479–496.

(3) Farrell, N. In *Polynuclear Platinum Drugs. Metal Ions in Biological Systems*; Sigel, A., Sigel, H., Eds.; Marcel Dekker, Inc.: New York, 2004; Vol. 41, pp 252–296.

(4) Hegmans, A.; Qu, Y.; Kelland, L. R.; Roberts, J. D.; Farrell, N. Novel approaches to polynuclear platinum pro-drugs. Selective release of cytotoxic platinum−spermidine species through hydrolytic cleavage of carbamates. *Inorg. Chem.* **2001**, *40*, 6108–6114.

(5) Rauter, H.; Di Domenico, R.; Menta, E.; Oliva, A.; Qu, Y.; Farrell, N. Selective platination of biologically relevant polyamines. Linear coordinating spermidine and spermine as amplifying linkers in dinuclear platinum complexes. *Inorg. Chem.* **1997**, *36*, 3919–3927.

(6) Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*; John Wiley & Sons: New York, 1999.

(7) Gerweck, L. E. Tumor pH: implications for treatment and novel drug design. *Semin. Radiat. Oncol.* **1998**, *8*, 176–182.

(8) Holley, R. W.; Holley, A. D. The removal of *N*-*o*-nitrophenoxyacetyl and *N*-chloroacetyl groups from peptides. *J. Am. Chem. Soc.* **1952**, *74*, 3069–3074.

(9) Masaki, M.; Kitahara, T.; Kurita, H.; Ohta, M. A new method for the removal of chloroacetyl groups. *J. Am. Chem. Soc.* **1968**, *90*, 4508–4509.

(10) Steglich, W.; Batz, H.-G. Removal of the chloroacetyl group from *N*-chloroacetyl derivatives of amino acids and peptides by means of 1-piperidinethiocarboxamide. *Angew. Chem., Int. Ed. Engl.* **1971**, *10*, 75–76.

(11) Kortüm, G.; Vogel, W.; Andrussow, K. *Dissociation Constants of Organic Acids in Aqueous Solution*; Butterworth & Co.: London, 1961.

(12) McGregor, T. D.; Hegmans, A.; Kasparkova, J.; Neplechova, K.; Novakova, O.; Penazova, H.; Vrana, O.; Brabec, V.; Farrell, N. A comparison of DNA binding profiles of dinuclear platinum compounds with polyamine linkers and the trinuclear platinum phase II clinical agent BBR3464. *J. Biol. Inorg. Chem.* **2002**, *7*, 397–404.

(13) Brabec, V.; Kašpárková, J.; Vrána, O.; Nováková, O.; Cox, J.; Qu, Y.; Farrell, N. DNA modifications by a novel bifunctional trinuclear platinum phase I anticancer agent. *Biochemistry* **1999**, *38*, 6781–6790.

(14) Moriarity, B.; Novakova, O.; Farrell, N.; Brabec, V.; Kasparkova, J. 1,2-GG intrastrand cross-link of antitumor dinuclear bifunctional platinum compound with spermidine linker inhibits DNA polymerization more effectively than the cross-link of conventional cisplatin. *Arch. Biochem. Biophys.* **2007**, *459*, 264–272.

(15) Roberts, J. D.; Peroutka, J.; Farrell, N. Cellular pharmacology of polynuclear platinum anti-cancer agents. *J. Inorg. Biochem.* **1999**, *77*, 51–57.

(16) Roffler, S. R.; Wang, S. M.; Chern, J. W.; Yeh, M. Y.; Tung, E. Antineoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody−enzyme conjugate. *Biochem. Pharmacol.* **1991**, *42*, 2062–2065.

(17) Bagshawe, K. D.; Sharma, S. K.; Springer, C. J.; Rogers, G. T. Antibody directed enzyme prodrug therapy (ADEPT): a review of some theoretical, experimental and clinical aspects. *Ann. Oncol.* **1994**, *5*, 879–891.

(18) Culver, K. W.; Ram, Z.; Wallbridge, S.; Ishij, H.; Oldfield, E. H.; Blaese, R. M. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* **1992**, *256*, 1550–1552.

(19) Takamiya, Y.; Short, M. P.; Ezzeddine, Z. D.; Moolten, F. L.; Breakefield, X. O.; Martuza, R. L. Gene therapy of maliganant brain tumors: a rat glioma line bearing the herpes simplex virus type 1-thymidine kinase gene and wild type retrovirus kills other tumor cells. *J. Neurosci. Res.* **1992**, *33*, 493–503.

(20) Brabec, V.; Palecek, E. Interaction of nucleic acids with electrically charged surfaces. II. Conformational changes in double-helical polynucleotides. *Biophys. Chem.* **1976**, *4*, 76–92.

(21) Kim, S. D.; Vrana, O.; Kleinwächter, V.; Niki, K.; Brabec, V. Polarographic determination of subnanogram quantities of free platinum in reaction mixture with DNA. *Anal. Lett.* **1990**, *23*, 1505–1518.

(22) Brabec, V.; Leng, M. DNA interstrand cross-links of trans-diamminedichloroplatinum(II) are preferentially formed between guanine and complementary cytosine residues. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5345–5349.

(23) Novakova, O.; Kasparkova, J.; Vrana, O.; van Vliet, P. M.; Reedijk, J.; Brabec, V. Correlation between cytotoxicity and DNA binding of polypyridyl ruthenium complexes. *Biochemistry* **1995**, *34*, 12369–12378.

(24) Kelland, L. R.; Jones, M.; Abel, G.; Valenti, M.; Gwynne, J.; Karrap, K. R. Human ovarian-carcinoma cell lines and companion xenografts: a disease-oriented approach to new platinum anticancer drug discovery. *Cancer Chemother. Pharmacol.* **1992**, *30*, 43–50.

(25) Kelland, L. R.; Barnard, C. F. J.; Mellish, K. J.; Jones, M.; Goddard, P. M.; Valenti, M.; Bryant, A.; Murrer, B. A.; Harrap, K. R. A novel *trans*-platinum coordination complex possessing *in vitro* and *in vivo* antitumor activity. *Cancer Res.* **1994**, *54*, 5618–5622.

(26) Montero, E. I.; Oehlsen, M. J.; Benedetti, B. T.; Mangrum, J. B.; Qu, Y.; Farrell, N. P. Pre-association of polynuclear platinum anticancer agents on a protein, human serum albumin *J. Chem. Soc., Dalton Trans.* **2007**, DOI: 10.1039/b708433c.

(27) Billecke, C.; Finniss, S.; Tahash, L.; Miller, C.; Mikkelsen, T.; Farrell, N. P.; Bögler, O. Sensitivity of glioma cells *in vitro* and *in vivo* to platinum anticancer drugs. Polynuclear agents are more potent than cisplatin and induce cell cycle arrest. *Neuro-oncology* **2006**, *8*, 215–226.

(28) Mitchell, C.; Kabolizadeh, P.; Ryan, J. J.; Roberts, J. D.; Yacoub, A.; Curiel, D. T.; Fisher, P. B.; Hagan, M. P.; Farrell, N. P.; Grant, S.; Dent, P. Low dose BBR3610 toxicity in colon cancer cells is p53-independent and enhanced by inhibition of ERBB1-PI3K signaling. *Mol. Pharmacol.* **2007**, *72*, 704–714.

(29) Nicoli, P.; Piazzoni, L.; Bai, A.; Torriani, D.; Pardi, G.; Grugni, M.; Natangelo, M.; Bastrup, U.; Allievi, C.; De Munari, S.; Di Giovine, S.; Cassin, M.; Pezzoni, G.; Singer, J. W. Novel bisplatinum complexes characterized by an improved *in vitro* plasma protein binding and *in vivo* efficacy on xenograft models. *Proc. AACR-NCI-EORTC* 2007, Published Abstract A267.

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