Effect of the Diamine Nonleaving Group in Platinum−Acridinylthiourea Conjugates on DNA Damage and Cytotoxicity

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The following complexes of type $[PtCl(R)(ACRAMTU)](NO_3)_2$ (ACRAMTU = 1-[2-(acridin-9-ylamino)-ethyl]-1,3-dimethylthiourea)), derived from prototype **1** (with R = ethane-1,2-diamine), were synthesized: **2** (with R = (1*R*,2*R*)-1,2-diaminocyclohexane), **3** (with R = propane-1,3-diamine), **4** (with R = N^1,N^1,N^2,N^2-tetramethylethane-1,2-diamine), and **5** (with R = 2,2′-bipyridine). The DNA sequence specificity of the conjugates and their antiproliferative potential in HL-60 and H460 cells were investigated. Conjugate **3** showed the strongest non-cisplatin-type DNA damage in polymerase stop assays and superior cell kill efficacy in H460 lung cancer (IC_{50} = 70 nM).

Introduction

Platinum−acridinylthiourea conjugates are a new class of cytotoxic DNA-targeted agents that act through a hybrid mechanism involving monofunctional platination of nucleobase nitrogen and classical intercalation.[1,2] This distinguishes this pharmacophore from clinical platinum agents, such as cisplatin (*cis*-$[PtCl_2(NH_3)_2]$), which act as cross-linking agents.[3] PT-ACRAMTU[a] (**1**), the prototype (Figure 1), produces monoadducts with guanine and adenine at sites dominated by the base step preference of the intercalator.[4,5] The most striking feature of PT-ACRAMTU's DNA reactivity proves to be its ability to induce adducts with adenine-N3.[6] The targeting of this donor site, which is located in the DNA minor groove, is unprecedented in platinum−DNA interactions and may be considered a new paradigm in metal-based chemotherapy.[7] PT-ACRAMTU shows promising biological activity superior to cisplatin in a wide range of solid tumor cell lines at (sub)micromolar concentrations,[8−11] especially in non-small-cell lung carcinomas characterized by various genetic backgrounds.[7]

To delineate structure−activity relationships within this class of compounds and to establish the structural requirements for the unusual adenine and minor-groove affinity of PT-ACRAMTU, we have made systematic changes to critical components of the prototypical agent. In previous work, we have studied the effects caused by structural modifications of the thiourea linker[8,11] and acridine moieties.[12] We have now generated a small library of complexes by replacing the en nonleaving group in the original design with the diamines (1*R*,2*R*)-1,2-diaminocyclohexane (dach), propane-1,3-diamine (pn), N^1,N^1,N^2,N^2-tetramethylethane-1,2-diamine (tmeda), and 2,2′-bipyridine (bpy) to afford the corresponding PT-ACRAMTU analogues **2**−**5** (Chart 1). Here, we demonstrate using cell proliferation assays and polymerase chain reaction based footprinting experiments that the nature of the diamine is of critical importance for the DNA reactivity and biological activity of these hybrid agents. Previous studies have used enzymatic digestion and base-selective depurination of globally platinated DNA, in conjunction with chromatographic separation and high-resolution methods (NMR, X-ray crystallography, MS), to determine the structures and relative abundances of adducts formed by PT-ACRAMTU. Although the footprinting technique does not provide any structural detail, it produces characteristic fingerprints of the damage pattern and is a more practical approach when comparing extended libraries of complexes.

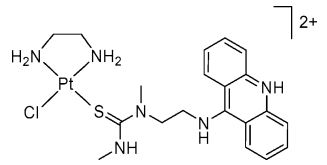

Figure 1. Structure of the drug prototype PT-ACRAMTU (**1**).

Results

Design and Chemistry. Bidentate en was used as a nonleaving group in PT-ACRAMTU to prevent substitution of the nitrogen ligand trans to thiourea sulfur and ultimately to eliminate the possibility of cross-link formation in cisplatin-specific poly-G tracts. Indeed, PT-ACRAMTU forms monoadducts in model systems and in native double-stranded DNA, and the formation of bifunctional adducts due to opening of the en chelate or loss of ACRAMTU has not been observed.[4] In the current study, four diamines were introduced to determine the effects of altered sterics, electronics, and H-bonding properties of the spectator ligand on the DNA base, sequence specificity of platination, and the biological activity of the conjugates. The dach and pn were chosen to vary chelate flexibility and steric bulk in the ligand periphery. The peralkylated derivative tmeda and aromatic bpy were introduced to

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[a] Abbreviations: PT-ACRAMTU, [PtCl(ethane-1,2-diamine)(1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea)]$(NO_3)_2$; ACRAMTU, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea; NSCLC, non-small-cell lung cancer; dach, (1*R*,2*R*)-1,2-diaminocyclohexane; pn, propane-1,3-diamine; tmeda, N^1,N^1,N^2,N^2-tetramethylethane-1,2-diamine; bpy, 2,2′-bipyridine; en, ethane-1,2-diamine; PCR, polymerase chain reaction; Taq pol, Taq DNA polymerase; d(d)NTP, 2′,(3′)-(di)deoxynucleoside-5′-triphosphate; bp, base pair; kb, 1000 base pairs.

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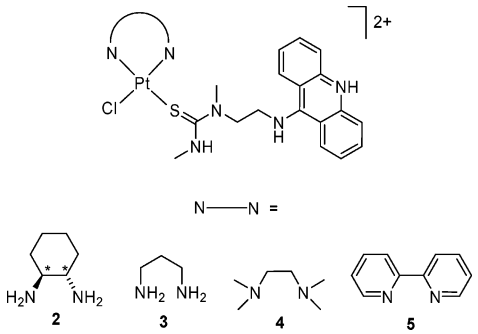
Chart 1. Structures of the PT-ACRAMTU Analogues Studied



eliminate the possibility of platinum−nucleobase H-bonding, a factor previously demonstrated to contribute to cisplatin's binding preference for guanine over adenine.[13]

The dicationic conjugates **2**−**5** were generated from the appropriate diaminedichloroplatinum(II) precursors after abstraction of one chloro ligand using silver ion followed by reaction with 1 equiv of ACRAMTU·HNO_3, according to the procedure developed for **1**.[11] All complexes were isolated as the water-soluble nitrate salts and fully characterized by ^{1}H NMR spectroscopy, elemental analyses, and electrospray mass spectrometry (see Experimental Section). The molecular structures were further confirmed by X-ray crystallography. Molecular views, selected geometric parameters, and crystal data for **2**−**5** have been deposited as Supporting Information.

DNA Damage. The sequence specificity of DNA adduct formation was monitored for conjugates **1**−**5** and cisplatin using a Taq pol inhibition assay in conjunction with linear signal amplification by PCR. In previous work, irreversible base damage resulting from alkylation and metalation has been studied using this method.[14,15] The assay is based on the extension of 5′ end-labeled primer oligonucleotides by Taq pol, which is prematurely terminated because of stalling of the enzyme at or near the DNA damage site, resulting in a band on the denaturing polyacrylamide sequencing gel. In this study, adduct formation was monitored in the 221-bp NdeI/HpaI fragment from plasmid pSP73 (Figure 2a), which was used previously by Leng et al. for mapping platinum−DNA adducts.[16] Briefly, **1**−**5** and the classical agent cisplatin were incubated with the restriction fragment at a drug-to-nucleotide ratio of 0.0075 at 37 °C for 48 h in the dark. Reactions with Taq pol were run unidirectionally using a 5′ end radiolabeled 25-bp primer (P1) with the bottom strand serving as the template, and the PCR products of the drug-modified DNA were analyzed on a sequencing gel alongside untreated control and dideoxy sequencing lanes (Figure 2b).

The most intense damage sites observed for **1** and cisplatin on the sequencing gel are summarized in Table 1. The hybrid agent produces adducts with G and A, specifically in sequences containing 5′-TA, 5′-GA/5′-AG, 5′-GT/5′-TG sites, which are completely absent from cisplatin's damage pattern (lanes 1 and 6 on the polyacrylamide gel in Figure 2b). These results are in complete agreement with previous transcriptional footprinting and enzymatic digestion/cleavage assays.[4,5,12] Characteristically, conjugate **1** produces stop sites in the sequence 5′-CCCC*G*GG, which appear to be localized to the first G following C, corroborating that platination is controlled by the drug's preferred intercalation into the 5′-CG/CG base-pair step.[17,18] This contrasts with the situation for cisplatin, which produces intense bands on the gel resulting from clusters of unresolved stop sites within G_n tracts (Figure 2b, Table 1), a consequence of the drug's tendency to induce 1,2 intrastrand cross-links between

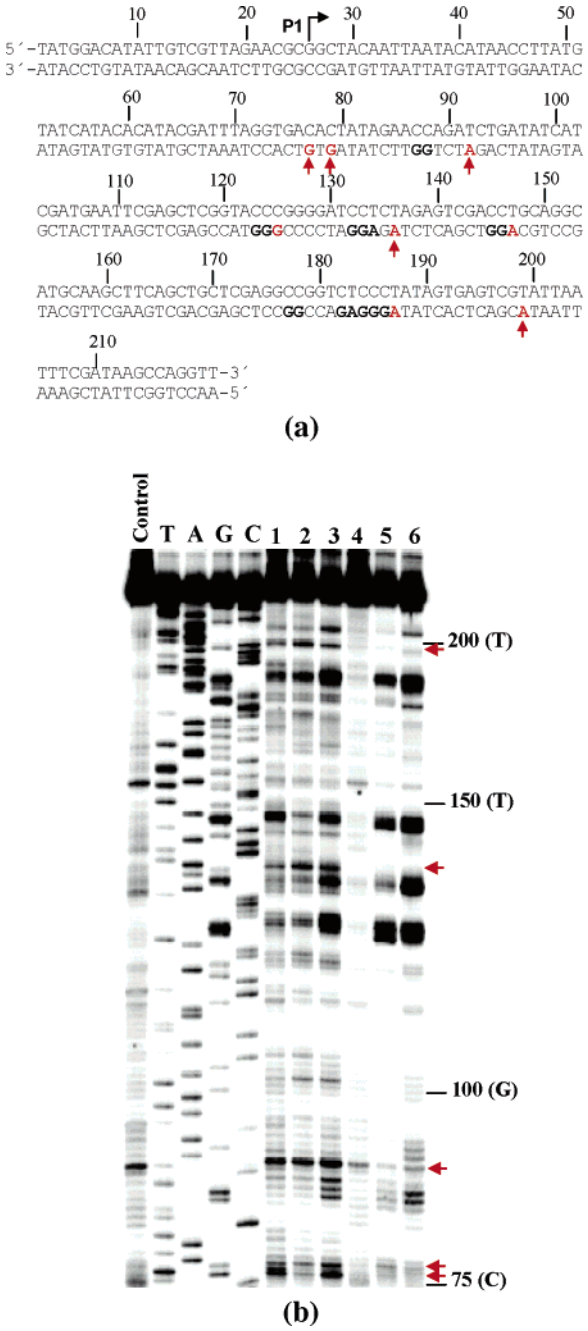


Figure 2. (a) Sequence of the 221-bp fragment used to study the base and sequence specificity of DNA damage caused by conjugates **1**−**5** and cisplatin. PT-ACRAMTU-specific and cisplatin-specific damages detected in the bottom strand through extension of primer P1 (bases 1−25) are highlighted with red and black bold letters, respectively. Characteristic intercalator-driven damage at nonconsecutive G and A bases is marked with red arrows. (b) Phosphorimage of the sequencing gel showing inhibition of primer extension by Taq DNA polymerase resulting from platination of nucleobases. Lane assignments (from left to right) are control, T, A, G, and C and are untreated damage control and dideoxy sequencing lanes, giving the sequence on the modified template (bottom) strand, which reads 5′ → 3′ from top to bottom of the gel. Adduct lanes 1−5 and 6 show the PCR products resulting from damage of the template by conjugates **1**−**5** and cisplatin, respectively. The bands highlighted with red arrows correspond to the damaged bases marked in (a).

consecutive purine bases.[3] It is noteworthy to mention that the damage detected in the present assay is in excellent agreement with that established in early work by Leng et al.,[16] which used SP6 RNA polymerase to map cisplatin−DNA adducts in the bottom strand of the same DNA fragment.

Table 1. Summary of PT-ACRAMTU-Specific and Cisplatin-Specific DNA Damage Detected by Enzymatic Footprinting[a]

PT-ACRAMTU (**1**)		cisplatin	
sequence context[b]	base no.	sequence context	base no.
A*GTGT*	80−76	T*GGT*	89−86
G*AT*	93−91	C*GGG*	126−123
C*GGG*	126−123	A*GGA*	134−131
T*AG*	137−135	*AGGT*	147−144
C*AGG*	148−145	C*GGC*	179−176
T*AG*	188−186	A*GGGAG*	187−182
T*AC*	200−198		

[a] Most intense damage based on densitometric band integration. [b] Sequences read 5′ → 3′. Apparent base damage is italicized.

Table 2. Cytotoxicity Data ($IC_{50} \pm$ SD μM) for **1**−**5**[a]

compd	R	HL-60 (leukemia)	H460 (lung)
1	en	2.8 ± 1.8[b]	0.26 ± 0.1[b]
2	dach	7.5 ± 0.9	1.3 ± 0.2
3	pn	3.9 ± 0.7	0.07 ± 0.02
4	tmeda	>100	>100
5	bpy	5.0 ± 0.2	1.6 ± 0.2

[a] MTS colorimetric cell proliferation assay. IC_{50} values were determined after 72 h of drug exposure and are averages of at least three experiments. [b] Reference 12.

Conjugates **2** and **3**, which contain dach and pn nonleaving groups, respectively, produce a damage pattern qualitatively similar to that of the prototype **1**. Differences, however, exist in the relative intensities of the stop sites among the three analogues. Several of the adducts formed by conjugate **2** result in significantly less intense bands on the gel (Figure 2b, lane 2) compared to the prototype (damage in the sequences CAGG, bases 148−145, and AGTGT, bases 80−76, Table 1, is negligible). In contrast, derivative **3** produces the most intense damage and additional stop sites at 5′-TGG (bases 89−87), which are virtually absent for all of the other conjugates tested. However, conjugates **4** and **5** show distinctly different behavior. The tmeda derivative **4** produces no stop sites on the gel. This is most likely a consequence of its inability to platinate nucleobase nitrogen because of the steric hindrance produced in the metal's coordination sphere by the bulky, peralkylated diamine. These results suggest that reversible intercalation alone is unable to stall polymerases, which has been demonstrated previously for the platinum-free acridines.[5] On the other hand, conjugate **5**, containing a planar diamine nonleaving group, produces an adduct profile that surprisingly resembles that of cisplatin rather than the damage produced by agents **1**−**3**. The compound appears to target runs of G bases, and the PT-ACRAMTU-specific damage at single A bases is virtually absent.

Cytotoxicity. The newly synthesized conjugates **2**−**5** were studied for their cytotoxic effect in human promyelocytic leukemia, HL-60, and human non-small-cell lung carcinoma cells, NCI-H460. The results of the cell proliferation assay are presented in Table 2. In HL-60, the new compounds showed moderate activity with IC_{50} in the low micromolar range, similar to prototypical **1**, with the exception of conjugate **4**, which did not show any dose response at concentrations as high as 100 μM. Replacement of the en nonleaving group with alternative diamines had a negligible effect on the biological activity of the hybrid agents in this cell line. All of the active conjugates show enhanced potency in the H460 cell line, and greater variation in IC_{50} is observed across the set of compounds tested compared to the HL-60 line, trends previously observed for PT-ACRAMTU analogues.[12] While a 10-fold enhancement in cytotoxicity is observed for PT-ACRAMTU (**1**) in the NSCLC line, an even more dramatic effect is observed for conjugate **3**, whose cell kill efficacy increases by approximately 55-fold in H460, based on the inhibitory concentrations calculated. The new derivative **3** inhibits cancer cell growth with an IC_{50} of 70 nM and proves to be the most potent conjugate identified to date. In contrast, analogues **2** and **5** exhibit only slightly enhanced cytotoxicity levels in the lung cell line, and the tmeda derivative **4** proves to be inactive.

Discussion

The steric bulk and stereochemistry of the nonleaving groups are important determinants of the reactivity and biological activity of cisplatin analogues. The presence of bulky diamines on platinum has a pronounced effect on the conformation of 1,2 intrastrand cross-links and the thermal stability of the damaged DNA.[19] Oxaliplatin, for instance, a second-generation cisplatin derivative, contains the bidentate nonleaving group dach instead of simple ammine (NH_3) ligands.[20] Its antitumor activity differs distinctly from that of other clinical platinum drugs, and it is being evaluated as a salvage therapy for cisplatin-resistant tumors.[20] Although it forms the same 1,2 intrastrand cross-links as cisplatin, even though at a slower rate, its adducts are more cytotoxic.[21] These differences in biological activity are thought to be caused, in part, by differential recognition and processing of the cisplatin and oxaliplatin GG adducts by repair proteins and DNA polymerases.[22] Nonleaving group effects in monoadducts formed by PT-ACRAMTU-type conjugates can be expected to have similar biological consequences.

In this paper we have studied the effects of variations in the diamine nonleaving group in PT-ACRAMTU analogues on DNA damage and biological activity. Derivatives **1**−**3** containing primary aliphatic diamines show overall similar adduct profiles but vary considerably in the intensity of the damage produced at specific sites. There appears to be a correlation between the nature of the diamine chelate, the DNA damage detected by enzymatic footprinting, and the cytotoxic effect observed in H460 lung cancer cells. (The HL-60 cell line appears to be less sensitive to all of the derivatives tested, and nonleaving group effects are minor, possibly indicating differences in cell kill mechanism in the two cell lines.) Conjugate **3**, which contains the conformationally most flexible (six-membered) chelate, produces the strongest damage and proves to be the biologically most active compound. Early kinetic studies on the aqueous chemistry of cisplatin analogues by House et al. have demonstrated that the nonleaving groups are effective modulators of complex reactivity.[23] Specifically, the authors demonstrated that expansion of the five-membered diamine chelate in $[PtCl_2(en)]$ by a single methylene group, giving $[PtCl_2(pn)]$, causes an approximate 2-fold increase in hydrolysis rates. We suggest that the intense DNA damage observed for **3** is the result of the platinum moiety's high reactivity and frequency of adduct formation, which may ultimately translate into increased cytotoxicity levels, as observed in the lung carcinoma cell line. Quantitative footprinting experiments are currently underway to study the kinetic aspects of DNA adduct formation by **1**−**3**. On the other hand, the footprinting and cytotoxicity data acquired for conjugate **2** suggest that the presence of the bulky cyclohexane residue may inhibit the formation of certain adducts, thereby decreasing its cell kill potential. In addition to these differences at the DNA binding level, other factors may also affect the biological activity of complexes **1**−**3**, such as differential cellular uptake due to changed polarity and lipophilicity of the platinum moieties.[24] Like the prototype **1**, complexes **2** and **3** retain the ability to form monofunctional

adducts with adenine, but it remains unclear if the introduction of the dach and pn nonleaving groups alters the groove specificity of platination at the damaged sites. Since adenine-N3 is a sterically crowded, less accessible nucleophilic site at the bottom of the minor groove, platination of this nitrogen can be expected to be favored by the flexible pn but disfavored by the bulky dach ligand.

To favor adenine binding over guanine binding by eliminating H-bond donor functions in the nonleaving groups, derivatives **4** and **5** were synthesized. Unfortunately, elimination of NH groups in permethylated tmeda in **4** introduces undesired steric bulk, which compromises the conjugate's ability to form adducts with nucleobase nitrogen, rendering the compound biologically inactive. These findings corroborate the notion that efficient coordinative binding of the metal moiety is a prerequisite for appreciable cytotoxicity in this class of agents. In an attempt to circumvent the steric hindrance associated with tertiary sp^3 nitrogen, planar bpy was introduced in derivative **5**. Curiously, this conjugate produces strong damage in poly-G tracts. However, attempts to detect guanine adducts of this drug in enzymatic digests of native DNA treated with conjugate **5** by in-line liquid chromatography−mass spectrometry and in model reactions with 2′-deoxyguanosine monitored by NMR spectroscopy were unsuccessful (unpublished data). We attribute this behavior to the lability of the adducts formed by **5** due to the strong trans-labilizing effect of the bpy ligand. Recently, Eldik et al. have demonstrated that aromatic (sp^2) nitrogen donors, which have pronounced π-acceptor properties, accelerate ligand exchange in platinum(II) complexes by up to 2 orders of magnitude compared to aliphatic (sp^3) amines.[25] Thus, it is possible that PT-ACRAMTU-type adenine adducts formed by conjugate **5** in the DNA major and minor grooves transform into *thermodynamically favored* guanine adducts in cisplatin-specific sequence contexts. The exact nature of these adducts, which are predicted to be more reactive than those formed by conjugates **1**−**3**, remains to be elucidated.

In conclusion, we have demonstrated that minor structural changes made to the nonleaving group in PT-ACRAMTU-derived agents may lead to an altered DNA damage profile and enhanced cytotoxicity. The results demonstrate that the spectator ligands in these conjugates are indeed critical modulators of biological activity. A minor structural change to the prototypical compound, namely, expansion of the en chelate by a single methylene group, resulted in the most active compound identified so far, conjugate **3**. Previous attempts to generate a conjugate with superior activity in the nanomolar range had failed. While changes to the acridine moiety, for instance, resulted in a significantly altered spectrum of DNA adducts, such derivatives were less cytotoxic properties than the prototype **1**.[12] Chemoresistance to platinum drugs is complex and multifactorial, and the design of novel first-line and salvage therapies is a high-priority goal in anticancer drug development.[26] Given the notorious insensitivity of NSCLC against DNA-targeted chemotherapeutic regimens, including platinum-based drugs and topoisomerase I poisons,[27] agents acting through dual mechanisms like the newly identified potent conjugate **3** may offer an advantage over existing treatments and should be pursued for further preclinical development. On the other hand, nonleaving group effects may also help circumvent several of the acquired resistance mechanisms. Future biological studies will therefore address the ability of the new compounds to overcome resistance in an extended panel of cell lines, including the previously studied ovarian cancers A2780/CP and C13*.[8−11]

Experimental Section

Materials and Product Characterization. Details of the product characterization and crystallographic details for 2−5 have been submitted as Supporting Information.

The following compounds were prepared according to previously described procedures: ACRAMTU, hydronitrate salt,[11] **1**,[11] and $[PtCl_2(bpy)]$.[28] $[PtCl_2(tmeda)]$, $[PtCl_2(dach)]$, and $[PtCl_2(pn)]$ were prepared using the method described by Dhara.[29]

A common procedure[11] previously developed for **1** was used, with minor variation, for the preparation of conjugates **2**−**5** using the appropriate diaminedichloroplatinum(II) precursor. A typical procedure is given for conjugate **2**.

[PtCl(dach)(ACRAMTU)](NO$_3$)$_2$·H$_2$O (2·H$_2$O). A mixture of 0.148 g (0.39 mmol) of $[PtCl_2(dach)]$ and 0.066 g (0.39 mmol) of $AgNO_3$ in 10 mL of anhydrous DMF was stirred at room temperature in the dark for 24 h. Precipitated AgCl was filtered off through a Celite pad, and 0.144 g (0.37 mmol) of ACRAMTU was added to the filtrate. The mixture was stirred for 12 h in the dark. DMF was removed in vacuum, and the oily residue was redissolved in a minimum of hot ethanol. The hot solution was treated with activated carbon, filtered through a Celite pad, and stored at 4 °C for 12 h. A bright-yellow precipitate of crude complex **2** was obtained, which was purified by repeated recrystallization (two to three times) from ethanol to give yellow crystals of **2**. Yield: 0.103 g, 35%. ^{1}H NMR (D_2O): δ 8.28 (2H, d, J = 8.7), 7.95 (2H, t, J = 7.8), 7.69 (2H, d, J = 8.6), 7.58 (2H, t, J = 7.8), 4.46 (4H, br s), 3.05 (3H, s), 2.86 (3H, s), 2.33 (2H, br s), 2.02 (2H, t, J = 11.4), 1.60 (2H, br s), 1.25−1.12 (4H, m). ESI-MS (MeOH, +ve mode) *m/z*: 669.1 $[M - H]^+$. Anal. ($C_{24}H_{35}N_8O_6SClPt·H_2O$) C, H, N.

[PtCl(pn)(ACRAMTU)](NO$_3$)$_2$ (3). Yield: 0.090 g (32%). ^{1}H NMR (D_2O): δ 8.23 (2H, d, J = 8.7), 7.94 (2H, t, J = 7.8), 7.63 (2H, d, J = 8.5), 7.57 (2H, t, J = 7.8), 4.39 (4H, br s), 3.12 (3H, s), 2.89 (3H, s), 2.75−2.70 (2H, m), 2.68−2.64 (2H, m), 1.81−1.74 (2H, m). ESI-MS (MeOH, +ve mode) *m/z*: 629.2 $[M - H]^+$. Anal. ($C_{21}H_{31}N_8O_6SClPt$) C, H, N.

[PtCl(tmeda)(ACRAMTU)](NO$_3$)$_2$ (4). Yield: 0.148 g (50%). ^{1}H NMR (D_2O): δ 8.28 (2H, d, J = 8.6), 7.95 (2H, t, J = 7.7), 7.7 (2H, d, J = 8.6), 7.58 (2H, t, J = 7.8), 4.46 (2H, t, J = 5.0), 4.26 (2H, br s), 3.03 (3H, s), 2.76−2.57 (19H, overlap, m). ESI-MS (MeOH, +ve mode) *m/z*: 671.2 $[M - H]^+$. Anal. ($C_{24}H_{37}N_8O_6SClPt$) C, H, N.

[PtCl(bpy)(ACRAMTU)](NO$_3$)$_2$·H$_2$O (5·H$_2$O). Yield: 0.092 g (30%). ^{1}H NMR (D_2O): δ 8.63 (1H, d, J = 5.7), 8.27 (1H, d, J = 5.8), 8.09−8.03 (3H, m), 7.94 (1H, t, J = 7.7), 7.79 (1H, d, J = 7.9), 7.7−7.63 (3H, m), 7.32−7.21 (6H, m), 4.63−4.6 (4H, m), 3.21 (3H, s), 3.11 (3H, s). ESI-MS (MeOH, +ve mode) *m/z*: 711.1 $[M - H]^+$. Anal. ($C_{28}H_{29}N_8O_6SClPt·H_2O$) C, H. N: calcd, 13.12; found, 12.61.

Enzymatic Footprinting. Generation of the Restriction Fragment. Enzymatic Footprinting. The generation of the 221-bp restriction fragment is described in Supporting Information.

Drug Incubations. Solutions of conjugates **1**−**5** and cisplatin were prepared in 10 mM Tris-HCl buffer (pH 8), and concentrations were determined by UV−vis spectrophotometry using ϵ_{413} = 9452 M^{-1} cm^{-1} for **1**,[1] ϵ_{416} = 9120, 9430, 8810, and 11 680 M^{-1} cm^{-1} for **2**−**5**, respectively, and ϵ_{300} = 132 M^{-1} cm^{-1} for cisplatin.[30] Appropriate concentrations of DNA (10 μg/50 μL ≈ 615 μM [nucleotides]) were incubated with the conjugates and cisplatin at r_f = 0.0075 (formal drug-to-nucleotide ratio) in 10 mM Tris-HCl buffer (pH 8.0) at 37 °C for 48 h in the dark. Drug-modified DNA samples were stored at −20 °C.

Linear Amplification Reactions. For each reaction, 20 pmol of primer 1 (P1) were 5′ end radiolabeled using 20 units of T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and 50 μCi of [γ-^{32}P]ATP (Amersham Biosciences, Piscataway, NJ). PCR for the platinum-damaged DNA was performed in DNA sequencing buffer using 100 μM each of dATP, dGTP, dCTP, dTTP (New England Biolabs, Ipswich, MA) precursors, 3.2 pmol of the labeled primer P1, and 5 units of Taq DNA polymerase (Promega,

Madison, WI) in a total reaction volume of 20 μL. The mixtures were subjected to five amplification cycles consisting of 30 s of denaturation at 95 °C, 30 s of annealing at 42 °C, and 60 s of polymerization at 70 °C. Dideoxy DNA sequencing reactions were performed using the dNTP/ddNTP mix supplied in the DNA sequencing kit according to the protocol provided by the vendor. The mixtures were subjected to 20 amplification cycles using the temperature program described above. Control reactions were performed for the unmodified template using the same conditions. Amplification reactions were performed in a BioRad DNA thermal cycler and terminated by adding an appropriate volume of DNA sequencing stop solution. The mixtures were electrophoresed on 8% polyacrylamide/8 M urea DNA denaturing sequencing gels. Samples were heated at 95 °C for 3 min, followed by rapid cooling on ice prior to electrophoresis. Gels were dried and analyzed on a Bio-Rad FX-Pro plus phosphorimager (Hercules, CA) using the Quantity One 1D analysis software (version 4.1.1; Bio-Rad Laboratories, Hercules, CA).

Cytotoxicity Assay. The cytotoxicity studies were carried out using the Celltiter 96 aqueous nonradioactive cell proliferation assay kit using a published protocol.[12] Stock solutions of **2**−**5** were prepared in phosphate-buffered saline. IC_{50} values were calculated from nonlinear fits using a sigmoidal dose response equation in GraphPad Prism (version 3.02; GraphPad Software Inc., San Diego, CA) and are averages of at least three individual experiments.

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Supporting Information Available: Analytical data, experimental and instrumental details, and results of the single-crystal X-ray structure determinations for **2**−**5**; crystallographic data in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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