Unique Base-Step Recognition by a Platinum−Acridinylthiourea Conjugate Leads to a DNA Damage Profile Complementary to That of the Anticancer Drug Cisplatin†

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ABSTRACT: The sequence specificity and time course of covalent DNA adduct formation of the novel platinum−acridine conjugate [PtCl(en)(ACRAMTU)]$(NO_3)_2$ [PT-ACRAMTU, **2**; en = ethane-1,2-diamine, ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea] have been investigated using restriction enzyme cleavage and transcription footprinting assays and compared to the damage produced by the clinical agent *cis*-diamminedichloroplatinum(II) (cisplatin, **1**). The rate of DNA binding of **1** and **2** was also monitored by atomic emission spectrometry. Restriction enzymes were chosen that cleave the phosphodiester linkage at, or adjacent to, the predicted damage sites. While conjugate **2** selectively protected supercoiled plasmid from cleavage by *Eco*RI and *Dra*I enzymes at their respective restriction sites, G↓AATTC and TTT↓AAA, **1** inhibited DNA hydrolysis by *Hin*dIII and *Psp*OMI at A↓AGCTT and G↓GGCCC (arrows mark cleavage sites) more efficiently. Transcription footprinting using T7 RNA polymerase revealed major single-base damage sites for **2** at adenine in 5′-TA and 5′-GA sequences. In addition, the enzyme is efficiently stalled at guanine bases, primarily in the sequence 5′-CGA where the damaged nucleobase is flanked by two high-affinity intercalation sites of ACRAMTU. While **1** targets poly(G) sequences, the binding of **2** appears to be dominated by the groove and sequence recognition of the intercalator. The biochemical assays used confirm previous structural information extracted from mass spectra of DNA fragments modified by **2** isolated from enzymatic digests [Barry, C. G., et al. (2003) *J. Am. Chem. Soc. 125*, 9629−9637]. Possible DNA-binding mechanisms and biological consequences of the unprecedented modification of alternating TA sequences by **2**, which occurred at a faster rate than binding to G, are discussed.

DNA-targeted cancer chemotherapies continue to play an important role in postgenomic drug discovery. The DNA sequence and groove specificity of adduct formation and the resulting structural changes within the biopolymer are critical determinants of the damage produced by these agents. DNA-binding proteins and/or DNA-processing enzymes, which also interact with DNA in a sequence- and groove-specific manner, are mediators of the cytotoxic effect produced by these agents (*1*). Therefore, one major goal in the design of new clinical agents is to produce new types of adducts on DNA, which may result in unprecedented downstream events in cell metabolism ultimately leading to apoptosis. *cis*-Diamminedichloroplatinum(II) [cisplatin[1] (**1**), Chart 1] is a potent anticancer agent thought to exert its biological activity through intrastrand cross-links formed in the major groove of DNA (*2*). Given the limitations of cisplatin-based chemotherapy, there is considerable interest in the discovery of new platinum-derived chemotypes that produce DNA adducts structurally different from those induced by the clinical agent (*3*).

Platinum(II)−acridinylthiourea conjugates are a novel class of DNA-targeted cytotoxic agents that show activity in a broad range of solid tumor cell lines (*4*−*6*). Unlike **1** and its derivatives, these compounds do not form purine−purine cross-links. The prototype, PT-ACRAMTU [**2**, nitrate salt, Chart 1; ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea], associates with DNA through a combination of *monofunctional* platination of nucleobase nitrogen and intercalation of the acridine nonleaving group into the DNA base stack (*7*). The most striking feature of this compound proved to be its ability to covalently modify adenine in approximately 20% of the adducts formed in DNA, which most likely involves binding to the N3 position of the base in the minor groove (*8*). We investigated the

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[1] Abbreviations: ACRAMTU, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea hydronitrate salt; bp, base pair; cisplatin, *cis*-diamminedichloroplatinum(II) [*cis*-$[PtCl_2(NH_3)_2]$]; dGuo, 2′-deoxyguanosine; en = ethane-1,2-diamine; 3′-dNTP, 3′-deoxyribonucleoside 5′-triphosphate; HPLC, high-performance liquid chromatography; ICP-AES, inductively coupled plasma atomic emission spectrometry; LC-MS, in-line liquid chromatography−mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MS/MS, tandem mass spectrometry; pMB109, a plasmid derived from pCR-Blunt II-TOPO (3519 bp; Zero Blunt TOPO PCR cloning kit; Invitrogen Life Technologies, Carlsbad, CA); nt, nucleotide; PT-ACRAMTU, [PtCl(en)(ACRAMTU-*S*)]$(NO_3)_2$; r_b, covalently bound platinum-to-DNA nucleotide ratio determined by ICP-AES; r_f, platinum-to-DNA nucleotide incubation ratio; TBP, TATA-binding protein.

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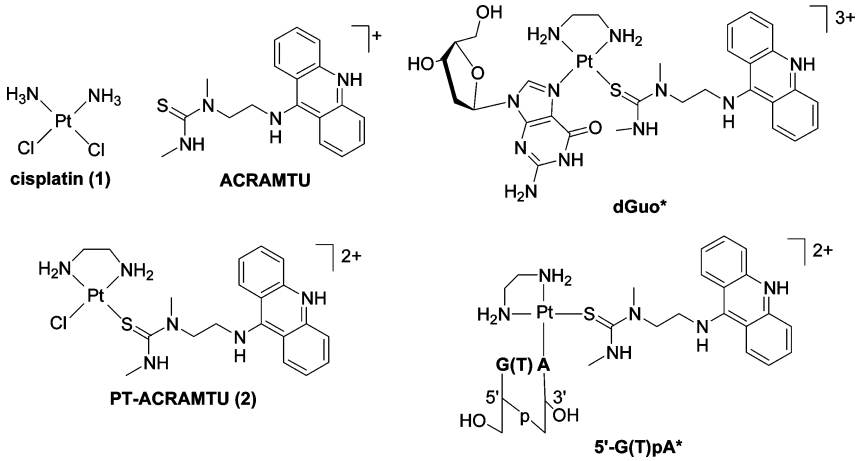
Chart 1: Structures of the Drugs and Adducts Formed by Conjugate **2** Identified in Enzymatic Digests



reactions of **2** with 2′-deoxyguanosine (dGuo) and random-sequence native DNA by in-line liquid chromatography/mass spectrometry (LC/MS) and NMR spectroscopy (^{1}H, ^{195}Pt) in order to identify the covalent adducts formed by platinum. In enzymatic digests of calf thymus DNA treated with **2**, three adducts were identified: [Pt(en)(ACRAMTU)(dGuo)]$^{3+}$ (dGuo*, **A1**, major), [Pt(en)(ACRAMTU){d(GpA)}]$^{2+}$ (**A2**, minor), and [Pt(en)(ACRAMTU){d(TpA)}]$^{2+}$ (**A3**, minor) (en = ethane-1,2-diamine) (Chart 1). The crystal structure of **A1** (chloride salt) has been solved recently (*9*). The Pt-N7-G coordination in this structure confirms major groove binding of platinum in approximately 80% of the lesions identified. **A1** has also been site-specifically incorporated into a synthetic dodecamer, d(5′-CCTCTCG*TCTCC-3′/3′-GGAGAGCAGAGG-5′), and characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), circular dichroism (CD) spectropolarimetry, UV melting curves, NMR spectroscopy, and molecular modeling (*10*). The combined data suggested that **A1** does not mimic the major cisplatin-induced DNA damage. In the dinucleotide adducts **A2** and **A3**, platinum binds to adenine at 5′-GA and 5′-TA base steps, which, along with 5′-CG, had previously been identified as high-affinity intercalation sites of ACRAMTU (*11*). **A2** and **A3** are the first examples of monofunctional adenine adducts of divalent platinum formed in double-stranded DNA. The selective targeting of adenine in the 5′-GA sequence by **2** is unprecedented and clearly distinguishes this agent from cisplatin-type compounds, which are known to induce cross-links preferentially at GG and 5′-AG (*12*). ACRAMTU has been demonstrated to intercalate into the 5′-GA/TC base step from the minor groove, suggesting that the covalent binding of platinum to nucleobase nitrogen in 5′-GpA* is driven by the sequence and groove specificity of the 9-aminoacridine derivative. The absence of platinated mononucleoside, A*, in enzymatic digests implies that the phosphodiester linkage in 5′-GpA* and 5′-TpA* is resistant to endonucleolytic cleavage (*8*). In this study we used restriction enzyme cleavage assays and in vitro transcriptional footprinting to further elucidate the differential DNA-damage profiles of **1** and **2**. Critical differences between these two agents emerge with respect to base and sequence specificity and kinetics of adduct formation, rendering conjugate **2** the first DNA-targeted platinum-containing agent that shows a DNA-binding profile truly complementary to that of the clinical drug cisplatin.

MATERIALS AND METHODS

Starting Materials. Cisplatin (**1**), ACRAMTU (hydronitrate salt), and PT-ACRAMTU (**2**) (nitrate salt) were prepared as desribed previously (*4*, 13). Stock solutions of **1** and **2** (226 μM in 10 mM Tris-HCl buffer, pH 7.5) were prepared at 25 °C and stored in the dark at −20 °C. The plasmid, pMB109 (3568 bp), was generated from the linearized plasmid, pCR-Blunt II-TOPO (3519 bp), by direct insertion into the vector of a blunt-end 49-bp sequence: 5′-CGATTAATTCGTTATCCCAGCTGTGAGTTCGACGATTAGAGCACTGGCC-3′ and 3′-GCTAATTAAGCAATAGGGTCGACACTCAAGCTGCTAATCTCGTGACCGG-5′. The complementary strands of the 49-bp insert were synthesized by Integrated DNA Technologies (Coralville, IA) using phosphoramidite chemistry and purified by HPLC. The strands were annealed in 100 mM NaCl by heating to 95 °C for 5 min followed by slow cooling to room temperature. The plasmid was constructed using the Zero Blunt TOPO PCR cloning kit from Invitrogen (Carlsbad, CA). All manipulations were carried out according to the instructions provided by the manufacturer. Plasmid DNA was isolated using a Plasmid Mega kit from Qiagen (Valencia, CA). The MAXIscript SP6/T7 kit for transcriptional footprinting was obtained from Ambion (Austin, TX), [α-^{32}P]UTP was from Perkin-Elmer (Boston, MA), 3′-dNTPs were from Trilink Biotechnologies (San Diego, CA), and all restriction endonucleases were from New England Biolabs (Beverly, MA).

Platination of DNA. For restriction enzyme cleavage assays, **1** and **2** were incubated with 40 nM plasmid at a drug-to-nucleotide ratio (r_f) of 0.1 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.9) at 37 °C. Platinum-modified DNA for transcription footprinting experiments was prepared using a plasmid concentration of 100 nM and incubation ratios of 0.01 and 0.005 for **1** and **2**, respectively. Samples of 100 μL were removed from the reaction mixtures after 1, 2, 6, 12, and 24 h, treated with 10 mM thiourea (*14*) for 30 min at 4 °C, and stored at −20 °C. All DNA samples were dialyzed for 48 h at 4 °C against 3 L of TE buffer using a 28-well microdialysis system from Gibco BRL (Gaithersburg, MD) prior to the incubations with restriction enzymes and polymerase. The platinum content was determined by

inductively coupled plasma atomic emission spectroscopy (ICP-AES) on a Perkin-Elmer Optima 3100XL ICP-emission spectrometer. Samples were decomposed in 2.5% HNO_3 at 55 °C for 24 h prior to ICP-AES analysis. Bound drug-to-nucleotide ratios (r_b) were calculated from molar platinum and DNA concentrations determined spectrophotometrically. The reported values are averages of three independent determinations. Platinum standard was from High-Purity Standards (Charleston, SC).

Restriction Endonuclease Assay. Assays were performed by incubating 2 pmol (5 μL) of each sample with 5 units of the appropriate endonuclease at 37 °C for 15 min followed by incubation with 10 mM NaCN to remove covalently bound drug from the DNA. Digested samples and control plasmid were analyzed by agarose gel (1.5%) electrophoresis. Gels were stained with ethidium bromide and documented using the Kodak Electrophoresis Documentation and Analysis System 290 (Rochester, NY).

Transcription Inhibition Assay. pMB109 plasmid linearized with *Bam*HI was used as transcription template. The DNA was incubated with **2** and ACRAMTU at $r_f = 0.005$, 0.01, and 0.025. Incubation was for 6 h in TE buffer at 37 °C. Transcription reactions were performed as described in Ambion's MAXIscript manual (Austin, TX). Transcripts were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (12% acrylamide, 8 M urea) followed by ethidium bromide staining.

Transcriptional Footprinting. Unidirectional RNA sequencing was performed as described previously (*15*, *16*) with the following modifications: Sequencing reactions contained 0.5 μg of linearized plasmid pMB109, 1 mM GTP, ATP, and CTP, 0.5 mM UTP, and 25 μCi of [α-^{32}P]UTP in 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, and 10 mM dithiothreitol (DTT). 3′-dATP (75 μM), 50 μM 3′-dGTP, 100 μM 3′-dCTP, or 25 μM 3′-dUTP was added as chain terminator. Transcription reactions were started by adding 10 units of T7 RNA polymerase followed by incubation at 37 °C for 45 min. Reactions were stopped by adding loading buffer supplied by Ambion (Austin, TX) and heating at 95 °C for 3 min. Samples were electrophoresed on 10% polyacrylamide gels containing 8 M urea, which were dried and analyzed on a Bio-Rad FX-Pro plus phosphorimager (Hercules, CA). Band intensities were determined using the Quantity One 1-D Analysis Software (version 4.1.1; Bio-Rad Laboratories, Hercules, CA).

RESULTS

Restriction endonucleases (*17*) as well as DNA (*18*) and RNA (*19*, *20*) polymerases have been successfully used as biochemical tools for probing the sequence specificity of DNA binders, such as platinum-containing anticancer drugs. DNA adducts interfere with these enzymes, which leads to inhibition of their DNA-cleavage and strand-extension activities, respectively. The magnitude of the inhibition determined in these assays depends on the frequency and/or severity of the structural insult to the biopolymer caused by a specific adduct. In the present study, we have used both types of enzymes to monitor the covalent DNA damage caused by cisplatin (**1**) and PT-ACRAMTU (**2**).

Restriction Enzyme Cleavage Assays and Kinetics of DNA Binding. The 3568-bp plasmid pMB109 contains two palindromic *Eco*RI recognition sequences and one recognition sequence for each of the other three enzymes used in this study, *Dra*I, *Hin*dIII, and *Psp*OMI. The recognition sites, where the arrows indicate the sites of hydrolytic cleavage, and their location on the plasmid are as follows: *Eco*RI, G↓AATTC, bases 325−330 and 392−397; *Dra*I, TTT↓AAA (blunt end), bases 2686−2691; *Hin*dIII, A↓AGCTT, bases 276−281; *Psp*OMI, G↓GGCCC, bases 443−448. To probe the differential binding of the two platinum agents, enzymes were chosen that cleave the phosphodiester linkage at, or adjacent to, the predicted damage sites (5′-GA and 5′-TA for conjugate **2** and 5′-AG and GG for **1**). Conjugates **1** and **2** were incubated with closed-circular plasmid at a formal platinum-to-nucleotide ratio (r_f) of 0.1 at 37 °C, pH 7.9. Aliquots of the reaction mixtures were removed after incubation with drug for 1, 2, 6, 12, and 24 h, quenched with thiourea, exhaustively dialyzed to remove unbound platinum, and incubated with enzyme. The cleavage products were analyzed by agarose gel electrophoresis (Figure 1), and the amount of covalently bound platinum (drug-to-nucleotide ratio, r_b) was determined for each time point by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Figure 2). In control experiments, all enzymes proved to be efficient at linearizing the supercoiled (form I) and nicked open-circular forms (form II) of the untreated plasmid (Figure

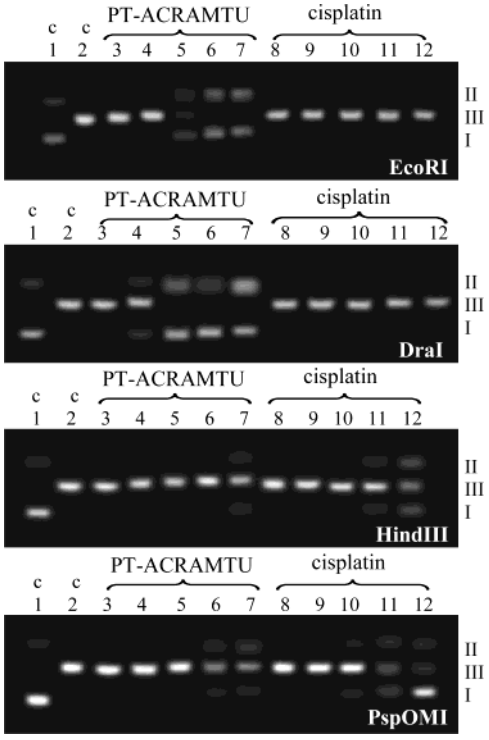


FIGURE 1: Restriction enzyme cleavage of pMB109 treated with platinum drug for various time intervals monitored by agarose gel electrophoresis. Control lanes: (1) untreated plasmid; (2) untreated plasmid cleaved with enzyme. PT-ACRAMTU lanes: (3) 1 h, r_b 0.013; (4) 2 h, r_b 0.050; (5) 6 h, r_b 0.092; (6) 12 h, r_b 0.094; (7) 24 h, r_b 0.094. Cisplatin lanes: (8) 1 h, r_b 0.004; (9) 2 h, 0.009; (10) 6 h, r_b 0.023; (11) 12 h, r_b 0.076; (12) 24 h, r_b 0.087. Bands are labeled I for supercoiled, II for nicked open circular, and III for linearized plasmid. To reverse cisplatin-induced cross-links and to avoid mobility shifts due to varying amounts of covalently bound drug, platinum was removed from the plasmid samples prior to electrophoresis using sodium cyanide (see Materials and Methods). Minor variations in the gel mobility and broadening of the bands are due to residual bound platinum in the samples.

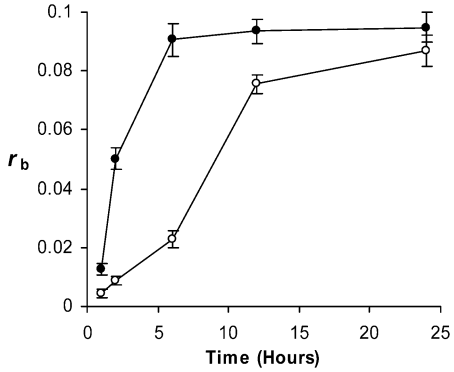


FIGURE 2: Degree of platination of pMB109 plasmid as a function of incubation time for **1** (open circles) and **2** (solid circles). Values are averages of three determinations.

1, lanes 2). Treatment of plasmid with **2** for 1 and 2 h produced a low degree of modification (r_b's of 0.013 and 0.050), which had no effect on the endonucleolytic activity of *Eco*RI. Only one band is observed for the linearized plasmid (form III), indicating efficient cleavage of the DNA. (Because the plasmid has two *Eco*RI sites, a 66-bp DNA fragment is released upon cleavage; this small fragment is not visible by ethidium staining because it has eluted off the gel.) At the 6 h time point ($r_b = 0.092$, lane 5), a major portion of form I and form II remains uncleaved, and the formation of form III is almost completely inhibited, based on the weak intensity of the corresponding band. In samples taken after 12 and 24 h, complete inhibition of *Eco*RI is observed (lanes 6 and 7). Cisplatin (**1**) had no effect on the cleavage activity of *Eco*RI, even at the highest platinum-to-nucleotide ratio reached after 24 h of incubation (lanes 8−12), which was comparable to the binding levels produced by **2** (see Figure 2). Cleavage assays using *Dra*I gave similar results (Figure 1). Inhibition of enzymatic cleavage at the TTTAAA restriction site by **2** was even more efficient than in the case of *Eco*RI: Small amounts of form I and form II plasmid were detected at a low r_b of 0.050 (lane 4). In contrast, plasmid cleavage by *Dra*I appears to be unaffected by **1**, even at high platinum-binding levels. The reverse situation is observed for *Hin*dIII and *Psp*OMI. For both enzymes, only minor cleavage inhibition by **2** could be established at the highest platinum-binding levels (form III produces the strongest bands). This is in contrast to **1**, which prevents the linearization of a considerable amount of forms I and II (lanes 11 and 12). On the basis of band intensities, both plasmids modified with **1** and **2** appear to be less susceptible to cleavage by *Psp*OMI than by *Hin*dIII. In the case of **1**, this difference most likely reflects the frequency of GG and AG adducts on the plasmid. [*cis*-[Pt(NH_3)$_2$-{d(pGpG-N7(1),N7(2))}] and *cis*-[Pt(NH_3)$_2$-{d(pApG-N7(1),N7(2))}] were identified previously (*21*) as the major (50%) and minor (28%) 1,2-intrastrand cross-links formed by the clinical drug in cell-free systems.]

A critical difference between **1** and **2** emerged with respect to the kinetics of adduct formation. While both compounds reach similar binding levels after 24 h of incubation, the time course of platination shows that the covalent modification of double-stranded DNA by **2** occurs more rapidly than modification by **1** and is virtually complete by 12 h. The extent of DNA damage sensed by the restriction endonucleases parallels platinum-binding levels observed by ICP-AES, suggesting that covalent modification of DNA is the cause of enzyme inhibition.

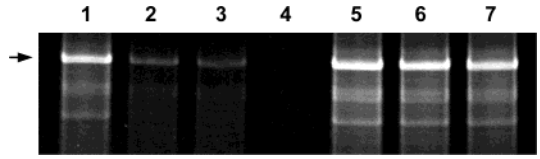


FIGURE 3: Transcription using T7 RNA polymerase of linearized untreated plasmid (lane 1) and linearized plasmid treated with conjugate **2** (lanes 2−4) and ACRAMTU (lanes 5−7). Formal drug-to-nucleotide ratios (r_f) were 0.005 (lanes 2 and 5), 0.01 (lanes 3 and 6), and 0.025 (lanes 4 and 7). Plasmids were incubated with drug for 6 h at 37 °C prior to the transcription assay. The arrow indicates the full-length product (121 nt) of runoff transcription.

Transcription Inhibition Assay. The inhibition of RNA polymerase-catalyzed transcription of drug-modified DNA leads to incomplete gene expression and is one of the potential mechanisms leading to cell death. To assess the effect of covalent adducts of conjugate **2** on runoff transcription of a globally modified DNA template and to establish enzyme−inhibitory concentrations for the transcriptional footprinting experiments (see below), *Bam*HI-linearized pMB109 was incubated with drug at various platinum-to-DNA nucleotide ratios (r_f). The ability of T7 RNA polymerase to transcribe the modified template was monitored by agarose gel electrophoresis (Figure 3). Conjugate **2** was able to reduce the amount of full-length product (121 nucleotides) significantly at r_f 0.005 and 0.01 based on the reduced band intensities for the full-length product compared to the untreated control. At r_f 0.025, transcription was completely inhibited (lanes 2−4, Figure 3). ACRAMTU itself did not cause noticeable inhibition over the whole concentration range (lanes 5−7, Figure 3), corroborating the fact that reversible intercalation alone is not sufficient to stall T7 RNA polymerase.

Transcriptional Footprinting. The ability of **1** and **2** to induce transcriptional blockages, i.e., premature termination of synthesis of the full-length RNA at, or in close proximity to, the adducts, was exploited to assess differences in the DNA adduct profiles of both compounds. Transcription products resulting from incubation of *Bam*HI-linearized, drug-modified pMB109 plasmid with T7 RNA polymerase were analyzed by polyacrylamide gel electrophoresis (Figure 4a) and quantified by densitometry (Table 1). The relevant portion of the sequence used to monitor the DNA damage including the T7 promoter region and the 49-bp insert (bases 337−385) is shown in Figure 5. The insert contained several of the predicted damage sites for **1** and **2**, based on previous studies. Transcription reactions were run unidirectionally from the T7 promoter region. Cisplatin (**1**) (lane 6 on the phosphorimage of the sequencing gel in Figure 4a) produced the expected major stop sites in runs of two or more purine bases (sites 1−5, Table 1), consistent with the formation of intrastrand cross-links in GG or 5′-AG dinucleotide sequences. Conjugate **1** also caused stop sites of lower intensity at 5′-GC and 5′-GA (sites 6−12, Table 1), where transcription is terminated either at G or at C. Damage at 5′-G**C** (where the bolded letter indicates the stop site) suggests that T7 RNA polymerase is stalled at a modified G on the opposite strand, most likely due to formation of the 1,2-interstrand cross-link (*20*). In contrast, PT-ACRAMTU (**2**)

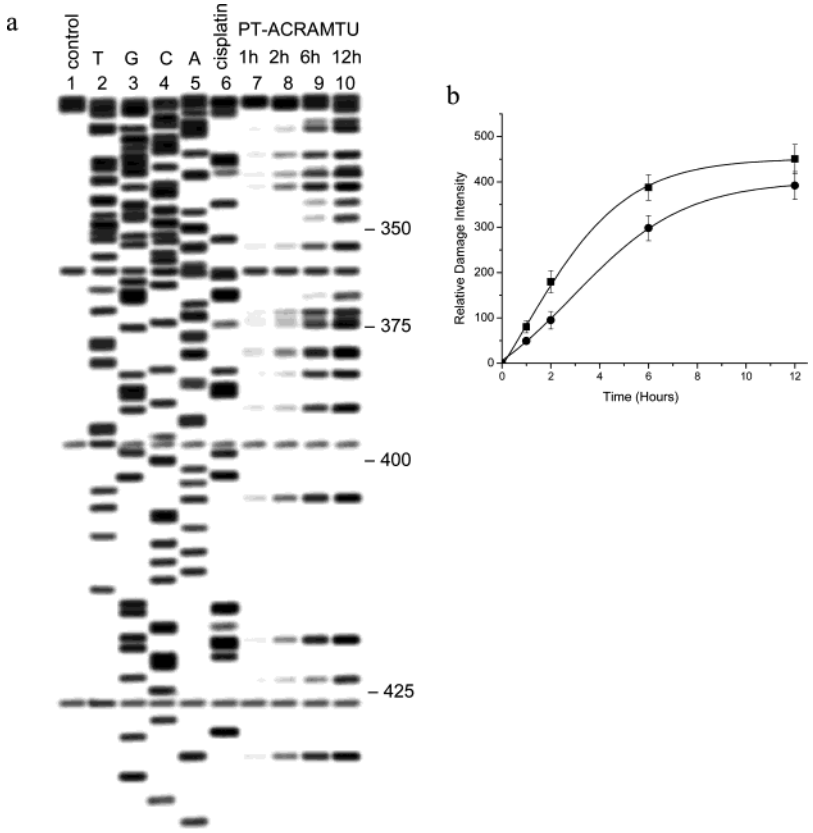


FIGURE 4: (a) Inhibition of RNA synthesis by T7 RNA polymerase of linearized pMB109 plasmid modified with cisplatin (**1**) and PT-ACRAMTU (**2**). Phosphorimage of a 10% polyacrylamide/8 M urea sequencing gel. Lanes: (1) transcribed unmodified template; (2−5) 3′-deoxynucleotide triphosphate (3′-dNTP) pMB109 sequencing lanes, giving the base sequence of the modified template strand; (6) transcribed plasmid incubated with **1** for 24 h; (7−10) transcribed plasmid incubated with **2** for the indicated time intervals (for r_b values see legend of Figure 1). The sequence of the template strand reads 5′ → 3′ from the top to the bottom of the gel. Transcription reactions of untreated DNA substrate resulted in several nonspecific stops common to all lanes. Such general stop sites could be caused by the secondary structure and/or degradation of the DNA template strand. (b) Time course of DNA damage involving A (squares) and G (circles). Relative intensities (arbitrary units) are averages of at least three resolved damage sites.

Table 1: Summary of Sequence-Specific DNA Damage by Cisplatin (**1**) and PT-ACRAMTU (**2**) in pMB109 Plasmid[a]

	cisplatin			PT-ACRAMTU		
site	rel intensity	sequence	base no.	rel intensity	sequence	base no.
1	1	***A**GGG*	387−390	1	TAAT	381, 382
2	0.85	C***GG***C	420, 421	0.88	GCGG	420
3	0.81	***GGG***A	368−370	0.84	ATAT	405
4	0.74	T***GG***C	417, 418	0.82	CGAA	376
5	0.65	C***AG***C	364, 365	0.75	CGAG	429
6	0.63	T***G***CA	399	0.68	CGAA	392
7	0.59	C***G***AG	428	0.65	CGAA	385
8	0.54	CA***G***A	402	0.63	ATAA	373
9	0.44	G***C***CG	422	0.51	CGAA	356
10	0.35	C***G***AA	385	0.38	CGCT	424
11	0.28	C***G***AA	376	0.18	GGAT	370
12	0.28	G***C***GG	419			

[a] Relative damage intensities (arbitrary units) were determined by densitometry of bands on the sequencing gel phosphorimage. Sequences are listed 5′ to 3′. Damaged bases are highlighted in italicized bold or underlined letters.

shows a damage profile in which the major stop sites observed for cisplatin are virtually absent (lanes 7−10, Figure 4a). Instead, the most intense damage for **2** is observed in the sequence TAAT (site 1, Table 1) where the stop site spans the adjacent adenines. All other stop sites involve a single A or G base. Strong to medium damage at adenine is also observed at ATAT, CGAG, and ATAA (Table 1), where the underlined base indicates the apparent site of transcription termination. Single guanines (G) appear to be the target of **2** in a 5′-CGN (N = A, G, C) sequence context, although one example was found where transcription is stalled at A following a 5′-CG base step (CGAG, site 5, Table 1). Overall, **1** and **2** appear to produce mutually exclusive DNA damage: While **1** targets multiple nucleobases in polypurine sequences, a damage pattern not observed for **2**, the conjugate produces adducts at 5′-TA, 5′-CG, and 5′-GA steps, which are not favored by **1**. Furthermore, damage sites of **2** are usually confined to *one* specific base. Although there is some overlap between the damage sites of the two agents (e.g., bases 368−370 and 417−422; Figure 5), conjugate **2** appears to target a single purine base in these sequences only in the presence of a flanking 5′-C or 3′-A base. Inhibition of T7 RNA polymerase by **2** occurs in a time-dependent manner as suggested by the increase in band intensities across lanes 7−10 (Figure 4a), which is additional proof that covalently bound platinum is the mediator of the enzyme inhibitory effect of **2**. An interesting effect is observed when comparing the relative averaged intensities for A- and G-specific damage (Figure 4b): The A adducts appear to form at a significantly faster rate than the G adducts. Possible explanations for this behavior would be higher affinity of acridine in **2** for A-containing base steps (*7*) and/or geometric factors, which

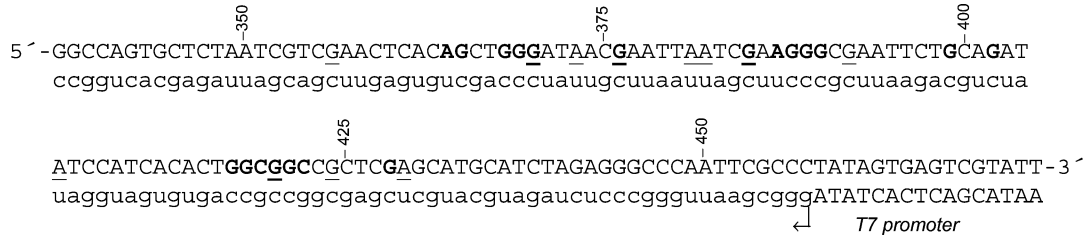


FIGURE 5: Section of the sequence used to monitor transcription of drug-modified pMB109 giving base numbering. Cisplatin damage sites are highlighted in bold letters. PT-ACRAMTU-specific damage is underlined.

may position platinum in favorable proximity to nucleophilic DNA donors.

DISCUSSION

DNA Base and Sequence Selectivity. The restriction enzyme cleavage assays and transcription mapping assays performed in this study confirm the nature of the DNA damage produced by cisplatin (**1**) reported previously (*2*). The DNA binding of **1** is driven by simple electrostatics (*22*), which directs the drug toward electron-rich poly(G) sequences in the major groove of DNA and favors cross-link formation at the metal's preferred nucleophilic target site, guanine N7. This is in contrast to PT-ACRAMTU (**2**), whose covalent interactions with DNA are dominated by the acridine's preference for intercalation at 5′-TA, 5′-GA, and 5′-CG sequences. The unique DNA recognition by this conjugate leads to platination of G or A at the preferred sites of intercalation of ACRAMTU (*11*). A closer examination of the damage profile indicates that platinum in **2** modifies purine base(s) on the 3′ side of base steps into which acridine intercalates. This can be clearly seen in the adenine-containing sequences 5′-TAAT (bases 380−383), 5′-ATAT (bases 403−406), and 5′-ATAA (bases 371−374) (Figure 5, Table 1). The reason for the damage at two consecutive adenines in the former sequence is unclear. Characteristically, 5′-AATT sequences are not a target for **2** (bases 377−880 and 393−396). A similar situation is observed for the 5′-CG sequence. In five of the damaged sites, **2** binds to G or A in the sequence 5′-CGA (Table 1). Platination of purine bases within this triplet may be favored due to the presence of two adjacent high-affinity intercalation sites, 5′-CG/CG and 5′-GA/TC (*11*).

Conjugate **2** is the first reported case of an intercalator-tethered complex of Pt^{2+} that produces DNA damage truly complementary to that of cisplatin. Other conjugates of the type intercalator−*cis*-Pt(diamine)Cl_2 containing a bifunctional metal exhibit DNA-binding profiles similar to that of cisplatin. Early studies by Bowler and Lippard using exonuclease III digestion demonstrated that acridine orange covalently tethered to cisplatinum complex, while promoting binding to CGG sites, produces the majority of adducts in poly(G) (*23*). 9-Anilinoacridines (*20*), acridinecarboxamides (*24*,*25*), phenazinecarboxamides (*26*), and phenanthridinium intercalators (*27*) reduce the sequence specificity of the *cis*-[$PtCl_2$] unit and cause additional damage and/or broadening of the damage sites but are unable to abolish cross-link formation in runs of consecutive G bases. An interesting feature is observed for the acridine- and phenazinecarboxamide complexes: the analogues in these series containing the shortest alkyl linker connecting the platinum and intercalator moieties showed the greatest differences in sequence specificity with cisplatin. This effect may be due to the reduced flexibility of the tether, which may, in analogy to our design rationale, prevent platinum from reaching poly(G) sites from the preintercalated state (*24*). Several major differences, however, exist between the platinum−acridinecarboxamides and conjugate **2**: (i) **2** contains a monofunctional platinum moiety, which eliminates the possibility of cross-link formation; (ii) ACRAMTU has a higher affinity for *adenine*-containing base steps, whereas the unmodified acridinecarboxamides show preferred intercalation into the 5′-CG/CG base step (*28*); (iii) of the two the 9-aminothiourea residue in ACRAMTU is located in the minor groove (*7*, *11*), while the residue in the 4-carboxamide derivatives protrudes into the major groove, where it makes specific hydrogen-bonding interactions with guanine N7 (*28*). The unique adduct profile of **2** may, therefore, be a consequence of the ability of ACRAMTU to hijack the non-cross-linking platinum moiety away from cisplatin-specific sequences in the major groove of DNA. Furthermore, the distinct groove specificity of the thiourea group in ACRAMTU may permit adduct formation at adenine in the minor groove, as has been suggested previously. Using a combination of enzymatic digestion and LC-MS/MS experiments, we observed platination of the 3′-A base in the unusual adduct 5′-GpA* (*8*). We argued that binding to this site might be favored in the minor groove in the absence of competing guanine N7, which is located in the major groove. One stop site of the 11 characterized was observed in the footprinting assays that strongly supports platination of A in this sequence, 5′-CG*A*G (site 5, Table 1). The majority of adducts (both in this study and by LC-MS/MS), however, appear to be formed with G in the major groove in the 5′-GA sequence and at virtually all 5′-CG sites. These findings suggest that even though the first DNA-binding step of **2** most likely involves intercalation from the minor groove, the regiospecificity of this interaction may not prevent the metal from reaching its nucleophilic target in the major groove, possibly due to threading of the Pt^{2+} moiety through the DNA base stack.

Time Course of DNA Binding. An early rationale for the design of DNA-targeted cisplatin was introduced by Denny et al. (*29*), who argued that the tethering of platinum to DNA-affinic ligands may have advantages for the pharmacokinetics of the drug by reducing its residence time in the cytosol, thereby limiting its exposure to detoxifying thiols and increasing the frequency of formation of cytotoxic adducts with DNA in the nucleus. Conjugates containing 9-anilinoacridine and acridine-4-carboxamides, inspired by the DNA affinity and biological activity of these intercalators, followed this strategy (see above). One of the major findings in cell-free systems was that the rate of platination was greatly enhanced for the DNA-targeted compounds compared to **1**, possibly indicating a direct substitution mechanism (*24*). The kinetically favored binding of 9-aminoacridine-4-carboxa-

mide analogues of cisplatin over **1** has been demonstrated recently to persist in whole cells (*25*). The time course of DNA platination observed in this study (Figure 2) demonstrates that platinum in conjugate **2** binds to double-stranded DNA at a faster rate than cisplatin (**1**). Interestingly, the opposite situation is observed in reactions of **2** with dGuo, whose slow binding has been ascribed to the increased steric hindrance of the bulky thiourea nonleaving group in **2** compared to the ligands in simple chloroam(m)ine complexes (*6*). These findings suggest that **2** reacts more efficiently from a preintercalated state, possibly via a direct pathway that does not require aquation of the Pt−Cl bond, the mechanism proposed by Temple et al. (*24*). In contrast, slow hydrolysis of chloride is known to be the rate-limiting step in reactions of **1** with DNA. The DNA binding of **1** requires hydrolysis of one chloro ligand ($t_{1/2}$ at 37 °C ≈ 2 h) giving cationic *cis*-$[PtCl(H_2O)(NH_3)_2]^+$, which associates with the negatively charged DNA backbone electrostatically prior to platination of guanine ($t_{1/2}$ at 37 °C ≈ 6 min) (*2*). Furthermore, it is important to note that the intercalator-enhanced platinum binding in the platinum−acridinecarboxamides occurs without noticeable adduct specificity (*24*). In contrast, the damage produced by conjugate **2** showed that A-containing sites are kinetically favored over G-containing sites (Figure 4). The enhanced DNA affinity of **2**, which parallels enzyme inhibitory effects observed in the biochemical assays, may have pharmacokinetic advantages over cisplatin in addition to the unique damage profile established in this study.

Implications of Sequence Selectivity. The biochemical assays used here clearly demonstrate that platinating compounds **1** and **2** produce mutually exclusive damage sites according to their complementary binding preferences. Thus, it seems likely that the promising biological activity of **2** (and its derivatives) (*4*−*6*) is mediated by adducts distinctly different from those thought to be relevant for the cytotoxic effect of cisplatin. The analysis of the adduct profiles suggests that the sequence and groove specificity of ACRAMTU plays an important role in the molecular recognition of DNA by **2**. In particular, the targeting of duplex DNA at alternating TA sequences is unprecedented in platinum antitumor chemistry and may produce a new class of suicidal lesions that complement the more typical guanine N7 adducts.

Alternating TA sequences are critical for transcription initiation of both prokaryotic and eukaryotic genes. The conserved TATA sequence (*30*) within the eukaryotic RNA polymerase II promoter serves as a binding site for RNA polymerase and transcription factors such as the TATA-binding protein (TBP) subunit of transcription factor IIID. We expect that covalent adducts arising from the novel platinum−acridine conjugate will impact binding of TBP and other TA-specific proteins in a manner distinct from cisplatin adducts (*31*−*33*). Binding of **2** to TA sequences may therefore lead to faulty or incomplete transcription, a potentially cytotoxic event.

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