DNA Minor Groove Adducts Formed by a Platinum−Acridine Conjugate Inhibit Association of TATA-Binding Protein with Its Cognate Sequence†

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ABSTRACT: PT-ACRAMTU ([PtCl(en)(ACRAMTU-*S*)]$(NO_3)_2$, en = ethane-1,2-diamine, ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea) is a cytotoxic platinum−acridine conjugate previously shown to form adducts with the N3 endocyclic nitrogen of adenine in the DNA minor groove. This unusual observation and our prior determination of the pronounced 5′-TA/TA base-step affinity of the drug have prompted us to investigate effects of these adducts on DNA minor groove binding proteins. Here, we used electrophoretic mobility shift assays to study the recognition of a PT-ACRAMTU-modified TATA box sequence by TATA-binding protein (TBP). The frequency of PT-ACRAMTU adducts in the minor groove of the TATA box was varied by selective elimination of potential major groove and minor groove binding sites in a 24-bp probe sequence through incorporation of deaza nucleobases. The most dramatic effect on TBP binding was observed in a duplex substituted with 7-deaza-G and 7-deaza-A, which reduced binding by as much as 73% compared to an unplatinated duplex. In contrast, elimination of A-N3 binding sites had no significant effect on TBP binding, suggesting that minor groove adducts of PT-ACRAMTU are the cause of inhibition. This notion was further corroborated by efficient platinum-mediated photo-cross-linking of the drug-modified DNA to TBP. PT-ACRAMTU appears to be the first platinum-based drug capable of targeting DNA sequences critical for transcription initiation. The biological consequences of PT-ACRAMTU's minor groove adducts are discussed.

DNA-targeted agents play an important role in cancer chemotherapy (*1*). Platinum-based drugs such as *cis*-diamminedichloroplatinum(II) (cisplatin,[1] Chart 1) and its derivatives are widely used as treatments for solid tumors (*2*). The anticancer activity of cisplatin-type therapies is attributed to the irreversible platination of purine N7 positions in the major groove of DNA, especially in adjacent guanine residues, leading to both intrastrand (major) and interstrand (minor) cross-links (*3*). The structural damage resulting from the 1,2-d(GpG) intrastrand cross-link, the putative cytotoxic lesion (*4*), is characterized by local unwinding of the modified duplex at the platinated base pair step. In addition, a sharp, directed bend of the duplex toward the major groove is observed, which leads to a concomitant widening of the minor groove (*5*−*9*). These structural alterations are believed to interfere with DNA-processing enzymes and DNA minor groove binding proteins to trigger downstream events ultimately leading to apoptotic cell death. Direct and indirect mechanisms for cytotoxicity have been proposed. Direct effects include inhibition of the replication and transcription machinery of the cell (*10*, *11*), while indirect effects likely involve the recognition of DNA damage by over 20 proteins including high mobility group (HMG) domain proteins and TATA-binding protein (TBP) (*12*−*14*). Several types of HMG domain proteins, including HMG1, HMG2, UBF, and SRY, recognize bent DNA and bind to DNA−cisplatin adducts with high affinity (*15*−*17*). It was suggested that HMG domain proteins could provide a shielding mechanism that protects cisplatin adducts from being removed by nucleotide excision repair (NER) (*18*). Furthermore, the "hijacking" of TBP from its natural promoter sequences to cisplatin damage sites has been proposed to potentially reduce normal gene transcription (*19*−*21*). Thus, both mechanisms at the DNA level may be considered mediators or enhancers of cisplatin's cytotoxic effect.

Although cisplatin is a very effective anticancer therapeutic, especially for treatment of testicular cancer (*22*), resistance often develops after chronic exposure to this compound. Resistance arises as a consequence of reduced cellular uptake, intracellular changes that prevent cisplatin from interacting with nuclear DNA, replicative bypass mechanisms, increased repair, and altered apoptotic pathways (*23*). The combination of acquired resistance and other side effects limits the effectiveness of cisplatin-based chemotherapy (*24*). Many new platinum-derived agents have therefore been synthesized, which exhibit decreased toxicity

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[1] Abbreviations: ACRAMTU, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea hydronitrate salt; Asn, asparagine; bp, base pair; cisplatin, *cis*-diamminedichloroplatinum(II) [*cis*-$[PtCl_2(NH_3)_2]$]; EMSA, electrophoretic mobility shift assay; ds, double stranded; en, ethane-1,2-diamine; enplatin, dichloro(ethane-1,2-diamine)platinum(II) [$[PtCl_2$-(en)]]; hTBP, human TATA-binding protein; Ile, isoleucine; Leu, leucine; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; Phe, phenylalanine; PT-ACRAMTU, [PtCl(en)(ACRAMTU-*S*)]$(NO_3)_2$; TBP, TATA-binding protein; Thr, threonine; Val, valine.

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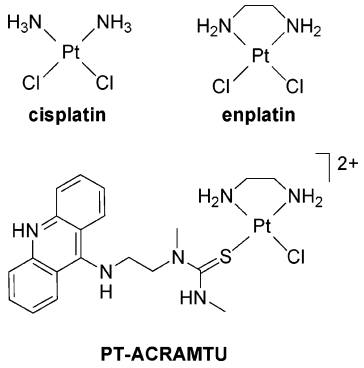
Chart 1: Structures of the Platinum Drugs Studied

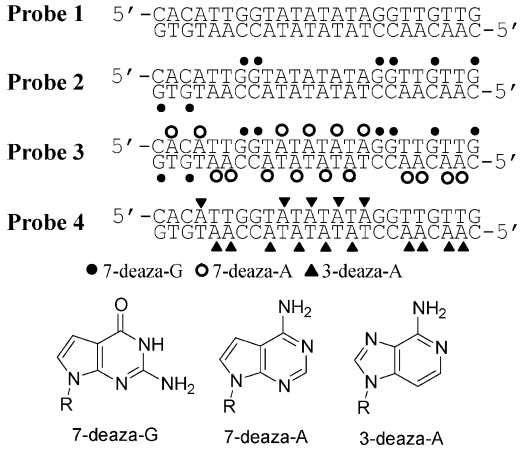


and presumably different mechanisms of action. Direct cisplatin derivatives, however, produce essentially the same kind of DNA damage and most likely trigger cell death via the same downstream events as the parent drug (*25*). Thus, it is crucial to design new agents that produce different DNA damage features, which might lead to alternative apoptotic mechanisms (*26*).

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 (*27*−*29*). PT-ACRAMTU ([PtCl(en)(ACRAMTU-*S*)]$(NO_3)_2$, Chart 1; en = ethane-1,2-diamine, ACRAMTU = 1-[2-(acridin-9-ylamino)-ethyl]-1,3-dimethylthiourea) modifies DNA through a dual mechanism involving intercalation and monofunctional platination but does not induce cross-links (*30*, *31*). A transcriptional footprinting study revealed that the binding preferences of this conjugate and cisplatin are mutually exclusive: while cisplatin predominantly targets 5′-GG and 5′-AG base steps (*4*), PT-ACRAMTU produces adducts with the 3′-purine base at 5′-TA, 5′-CG, and 5′-GA sites, which are unknown for cisplatin (*32*, *33*). Furthermore, we demonstrated that the unique binding profile of PT-ACRAMTU is dominated by the sequence and groove specificity of the ACRAMTU moiety (*34*), which has the effect of directing platinum away from cisplatin-specific sequences. Most strikingly, the distinct groove specificity of the thiourea group in ACRAMTU leads to platination of adenine-N3 in the minor groove of DNA (*35*). Minor groove adenine adducts appear to comprise a significant percentage (∼5−10%) of the total array of adducts established in previous studies (*33*).

Alternating TA motifs, a major target of PT-ACRAMTU, are the hallmark of promoter sequences responsible for recruiting eukaryotic RNA polymerase II. Assembly of the essential transcription initiation complex involves recognition of the TATA box sequence by the TBP subunit of transcription factor IID. Since TBP−TATA box binding is a key step in complex formation (*36*), alterations within the TATA box sequence may profoundly impact transcription (*37*). We previously argued that blocking TBP binding sites by bulky minor groove PT-ACRAMTU adducts could inhibit transcription, thereby affecting cell viability (*32*, *35*). Here we demonstrate using electrophoretic mobility shift assays (EMSA) and drug-mediated photo-cross-linking experiments that PT-ACRAMTU adducts localized to adenine-N3 in the minor groove of a palindromic $(TA)_4$ sequence dramatically reduce TBP binding. TATA box mediated transcription initiation emerges as a potential direct biological target for PT-ACRAMTU previously unknown in platinum antitumor chemistry.

Chart 2



MATERIALS AND METHODS

Preparation of Platinum Compounds. *cis*-Diamminedichloroplatinum(II), *cis*-$[PtCl_2(NH_3)_2]$ (cisplatin), PT-ACRAMTU (dinitrate salt), and dichloroethylenediamineplatinum(II), $[PtCl_2(en)]$ (enplatin), were prepared according to literature procedures (*29*, *38*). Stock solutions of cisplatin and PT-ACRAMTU (100 μM in 10 mM Tris-HCl buffer, pH 7.5) were prepared at 25 °C and stored in the dark at −20 °C. The activated, water-soluble form of $[PtCl_2(en)]$, $[Pt(en)(H_2O)_2](NO_3)_2$, was prepared by reacting $[PtCl_2(en)]$ with 1.95 equiv of $AgNO_3$ in water for 20 h followed by removal of the AgCl precipitate by filtration.

Oligonucleotide Probes. The unsubstituted oligonucleotide probe (probe 1, Chart 2) and the nucleobase analogue substituted probes (probes 2−4, Chart 2) were synthesized on an automated Applied Biosystems Model 394 DNA/RNA synthesizer using standard phosphoramidite chemistry. The phosphoramidite precursors of the nucleobase analogues 7-deaza-2′-deoxyguanosine (7-deaza-G), 7-deaza-2′-deoxyadenosine (7-deaza-A), and 3-deaza-2′-deoxyadenosine (3-deaza-A) were purchased from Glen Research (Sterling, VA). The oligonucleotides were purified by polyacrylamide gel electrophoresis (16%, 19:1 acrylamide:bisacrylamide/8 M urea) in 1 × TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Bands corresponding to full-length deprotected oligonucleotides were excised and electroeluted using the Elutrap system (Schleicher & Schuell, Dassel, Germany) followed by ethanol precipitation. Aqueous stock solutions of the oligonucleotides (100 μM in 10 mM Tris-HCl, pH 7.5) were stored at −20 °C until used.

5′-End Labeling of Oligonucleotides. The top strand of each probe (50 μM oligonucleotide) was radiolabeled at the 5′-end using T4 polynucleotide kinase (Promega, Madison, WI) and [γ-^{32}P]ATP (Perkin-Elmer, Billerica, MA). Radiolabeled oligonucleotides were purified using Sephadex G-25 Quick Spin columns (Roche, Indianapolis, IN) according to the manufacturer's protocol.

Platination of Duplexes. Prior to the platination reactions, the single-stranded oligonucleotide probes were annealed with their complementary strands (Chart 2) by heating both strands to 98 °C for 5 min followed by slow cooling to room temperature in a dry bath. Reactions were performed in 10

mM Tris-HCl buffer (pH 7.5) with duplex concentrations of 50 μM for unlabeled probes (used in CD and thermal melting experiments) and 1 μM for radioactively labeled probes. The labeled and unlabeled probes 1−4 were incubated with 0.1, 0.5, 1, 2, 3, and 4 molar equiv (drug:duplex) of PT-ACRAMTU. Cisplatin was incubated with the radioactively labeled probes 1−4 using 2 molar equiv of platinum complex. The reactions were incubated at room temperature for 48 h in the dark for both labeled and unlabeled probes. The mixtures containing unlabeled probes were treated with 10 mM thiourea for 30 min at 4 °C and subsequently dialyzed against 10 mM Tris-HCl buffer (pH 7.5) for 48 h. [Pt(en)-$(H_2O)_2$]$(NO_3)_2$ was reacted with radioactively labeled probe 1 at 0.1, 0.5, 1, 2, 3, and 4 molar equiv of platinum complex. MALDI-TOF mass spectra of the PT-ACRAMTU-modified probes were recorded by HT Laboratories (San Diego, CA) on a Voyager DE instrument (Applied Biosystems, Foster City, CA).

Electrophoretic Mobility Shift Assays. To generate TBP−TATA box complexes, radioactively labeled duplexes (final concentration of 15 nM) were added to a buffer containing 4% glycerol, 20 mM Tris-HCl (pH 8), 60 mM KCl, 5 mM $MgCl_2$, 100 μg/mL BSA, 1 mM DTT, and 125 ng of poly-(dG-dC)$_2$ (20 μL). The reactions were equilibrated at room temperature for 5 min before human TATA-binding protein (hTBP) was added to a final concentration of 3 nM, and incubation was continued for another 40 min. The hTBP used in the reaction (Protein One, College Park, MD) had a molecular mass of approximately 45 kDa, according to the manufacturer. Electrophoretic mobility shift assays (EMSA) were performed on 6% polyacrylamide gels (60:1 acrylamide:bisacrylamide) containing 25 mM Tris-HCl, 190 mM glycine, 2.5% glycerol, and 500 μM DTT. Gel loading buffer containing 80% glycerol, 50 mM Tris-HCl, 400 mM glycine, 0.05% bromophenol blue, and 0.05% xylene cyanol was added to the samples prior to the electrophoresis. Electrophoresis was carried out at 160 V for 30 min, and the gels were dried and analyzed on a Bio-Rad FX-Pro Plus phosphorimager (Hercules, CA). Bands on the gels were integrated using the Quantity One software (version 4.4.1; Bio-Rad, Hercules, CA). Reported intensities are averages of three independent experiments.

Determination of Dissociation Constants. Dissociation constants (K_d) were determined by adding varying amounts of hTBP to the platinated duplexes, and binding was quantified by EMSA as described above. K_d values were extracted from plots of the fraction of bound oligonucleotide against the hTBP concentration (nM). Data were fitted using a hyperbolic function in Origin 7 (Northampton, MA). The hTBP dissociation constants were determined for probes 3 and 4 modified with PT-ACRAMTU at a drug-to-probe ratio of 0.5.

Photo-Cross-Linking of Platinated Duplexes to hTBP. Binding reactions of hTBP with platinated duplexes (20 μL) were performed as described above for the mobility shift assay. Probes 3 and 4, modified with 0.5 equiv of PT-ACRAMTU, were used in this experiment. The final concentrations of hTBP in the mixtures were 9.0 and 3.8 nM for probes 3 and 4, respectively. This concentration of protein was calculated from the dissociation constants to yield approximately 60% bound oligonucleotide. After incubation for 40 min, the mixtures were placed on ice and irradiated from above to induce cross-link formation. Irradiation was carried out using a Transluminator Model 88 (Fisher Scientific, Pittsburgh, PA) operating at 312 nm for 90 min with 5 μL aliquots of sample removed every 30 min. Dark controls were also performed by heating the mixtures at 55 °C for 90 min. Irradiated and nonirradiated samples were combined with gel loading buffer containing 10 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanol, heated to 95 °C for 5 min, immediately placed on ice for 5 min, and subjected to 10% PAGE under denaturing conditions. The gels were then dried and analyzed by phosphorimaging.

Thermal Melting Studies. Melting studies were performed on an HP-8453 Hewlett-Packard UV−vis spectrophotometer equipped with an HP 89090A Peltier temperature control. Absorbances were measured at 260 nm every 1 °C from 15 to 90 °C and back to 15 °C with a heating/cooling rate of 0.5 °C/min and 1 min holding time. The studies were carried out in a buffer containing 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl. The melting temperatures were extracted from plots of relative absorbance versus temperature using the van't Hoff two-state model as described previously (*39*).

Circular Dichroism Specroscopy. Circular dichroism (CD) spectra were collected on an Aviv Model 215 CD spectropolarimeter in the range of 220−320 nm at 25 °C. The measurements were performed in 25 mM HEPES−KOH (pH 7.5) and 50 mM NaCl. CD data were transformed to molar ellipticity ($\Delta\epsilon$) as described in the literature by taking into consideration the oligonucleotide duplex concentrations (*40*).

RESULTS

Design, Platination, and Characterization of the Duplex DNA Probes. The DNA probes are 24-bp duplexes containing a palindromic TATA box element flanked by two GG sequences (probes 1−4, Chart 2). Previous crystal structure studies of TBP−TATA box complexes demonstrated that model duplexes as short as dodecamers are sufficient for TBP binding (*41*). We used 24-bp duplexes to enhance the thermal stability at room temperature of our TA-rich sequence and to avoid the formation of non-double strand specific adducts by PT-ACRAMTU. The TATA element selected (TATATA-TA) corresponds to the adenovirus E4 TATA box promoter sequence, which represents a high-affinity site for TBP binding (*41*). This sequence contains eight 5′-TA sites, the maximum possible number of 5′-TA target sites for PT-ACRAMTU within the TATA box (*32*−*34*), promoting efficient targeting of the drug to TBP's cognate sequence. The flanking GG dinucleotides were introduced to compare the differential effects on TBP binding of PT-ACRAMTU and cisplatin-modified DNA (*19*, *20*). Other sequences targeted by PT-ACRAMTU [5′-GA and 5′-CG (*32*)] were eliminated from the probe to minimize competitive binding.

In previous work we demonstrated that adenine adducts account for approximately 20% of the total DNA modifications arising from PT-ACRAMTU, with platination occurring at more than one nucleophilic donor site of adenine (*33*, *35*). As we sought to probe the effect of minor groove PT-ACRAMTU adducts of adenine, critical competing donor sites (A-N7 and G-N7) were eliminated from the duplexes by globally substituting adenine and guanine with N7-deazaadenine and N7-deazaguanine (probes 2 and 3). Conversely, to direct platinum away from the minor groove, a

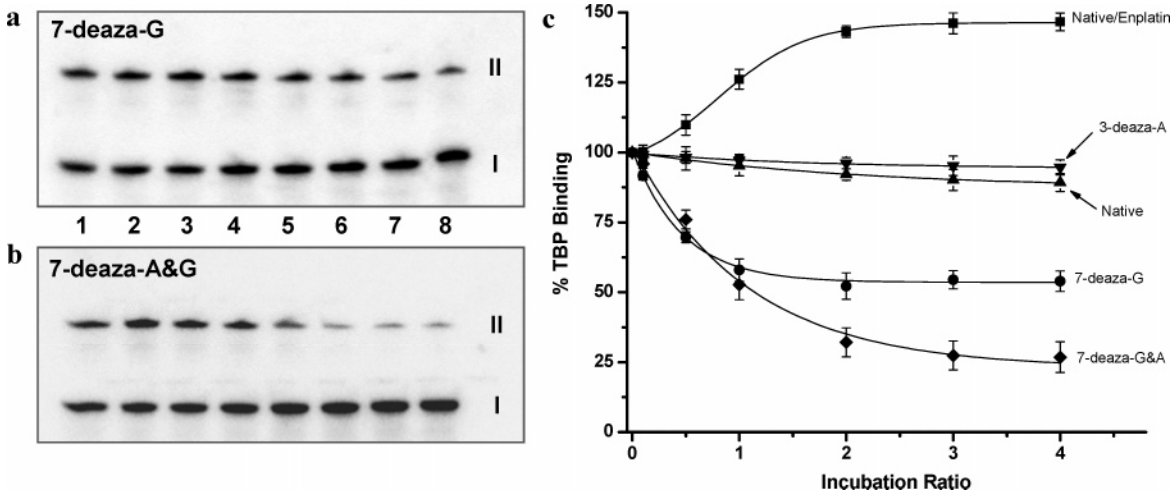


FIGURE 1: Binding of hTBP to drug-modified 24-bp duplexes at varying degrees of platination monitored by polyacrylamide gel electrophoresis. EMSA of hTBP binding to probes 2 (7-deaza-G; panel a) and 3 (7-deaza-A, 7-deaza-G; panel b). Lanes: (1) probe modified with 2 equiv of cisplatin; (2) unplatinated probe; (3−8) PT-ACRAMTU-modified probe at drug-to-duplex ratios of 0. 1:1, 0.5:1, 1:1, 2:1, 3:1, and 4:1, respectively. (c) Plot of hTBP binding levels vs incubation ratios. Inhibition (and enhancement) levels are relative to unmodified duplexes. Each data point is an average of three independent experiments, and error bars represent ±1 standard deviation.

probe containing N3-deazaadenine (probe 4) was also synthesized. To verify that incorporation of the unnatural nucleotides had no significant effect on DNA thermal stability and global conformation, we recorded UV melting curves and CD spectra of the modified and unmodified DNA duplexes. We observed melting temperatures of 319.4 and 320.1 K for the unplatinated probes 1 and 3, respectively, indicating that introduction of the deaza analogues has no significant effect on the thermal stability (Figure S1; see Supporting Information). Treatment of the two probes with PT-ACRAMTU resulted in increased melting temperatures of 8−9 K (Figure S1), in agreement with a monofunctional coordinative-intercalative binding mode established in previous studies of site-specifically platinated model duplexes (*30*, *42*). Probe 3 also maintains B-form structure, both in its platinum-free and platinated form (*30*, *43*): at the highest degree of platination, this duplex showed a negative band at 250 nm and a positive band at 275 nm, with a characteristic red shift of 2−3 nm compared to the unplatinated probe (*30*) (Figure S1). To assess the degree of platination within a modified TATA box, MALDI-TOF mass spectra were recorded for probe 3 treated with 0.5 and 4 molar equiv of PT-ACRAMTU (drug:duplex). Platination with 0.5 molar equiv of drug produces at least one adduct per double-stranded probe, while 4 molar equiv of drug generates a minimum of three adducts per duplex (Figure S2).

TBP Binding to the TATA Box Probes. Electrophoretic mobility shift assay (EMSA) analyses were performed to monitor the consequences of PT-ACRAMTU modification on TBP binding. Probes 1−4 were treated with varying amounts of PT-ACRAMTU and incubated with TBP prior to electrophoresis. At the highest incubation ratio (4 molar equiv of PT-ACRAMTU per duplex), TBP binding to the native duplex (probe 1) is reduced by approximately 11% (Figure S3). At the same degree of modification, elimination of nonspecific G-N7 binding in probe 2 results in a 46% inhibition of TBP binding (Figure 1a). Elimination of both G-N7 *and* A-N7 potential binding sites (probe 3) inhibits TBP binding even more dramatically, resulting in a 73% decrease in DNA−protein association (Figure 1b). As demonstrated by MALDI-TOF mass spectrometry analysis, platination of probe 3 with 4 molar equiv of PT-ACRAMTU produces probe duplexes containing *at least three* platinum adducts per TATA box (see Figure S2) which are distributed within the TATA box. This statistical distribution of PT-ACRAMTU among the eight potential binding sites likely induces partial binding of TBP to the TATA box, accounting for 27% TBP binding to platinated probe 3 instead of a total inhibition of TBP binding. For probes 2 and 3, cisplatin has no effect on the binding affinity of TBP, which is consistent with the absence of G-N7 sites in these duplexes. When minor groove binding of PT-ACRAMTU is prevented by incorporation of 3-deazaadenine in probe 4, TBP binding is reduced by only approximately 6% (Figure S3). This inhibition of binding is less than the 11% reduction observed for the native duplex (probe 1) with its full complement of major and minor groove sites available for adduct formation, establishing that inhibition of TBP binding correlates with the drug's ability to platinate A-N3 in the minor groove. In contrast, modification of probe 1 with classical enplatin enhances TBP binding approximately 1.5-fold (Figure S3), in agreement with prior studies of cisplatin damage by Lippard et al. (*19*, *20*). A graphical presentation of the probe-specific inhibition levels for various concentrations of platinum agent is shown in Figure 1c.

Photo-Cross-Linking of TBP to Platinum-Modified DNA. The interaction between a TATA box element and TBP is distinct from most protein−DNA interactions in that protein binding occurs in the minor groove, as shown in X-ray crystal structures of TBP from diverse species bound to their respective TATA boxes (*44*−*46*). Likewise, PT-ACRAMTU also intercalates and binds to N3 of adenine from the minor groove (*35*). On the basis of the molecular mechanism of TBP−DNA recognition (*41*) we reasoned that at some level of platination it should be possible for TBP and PT-ACRAMTU to occupy the same TATA box, potentially placing TBP in close proximity to the PT-ACRAMTU adduct (see also Discussion). As demonstrated by MALDI-TOF mass spectrometry analysis, platination of probe 3 with 0.5 molar equiv of PT-ACRAMTU primarily produces probe duplexes containing *one* platinum adduct per TATA box (see Figure S2). At this ratio, TBP binding is approximately 25%

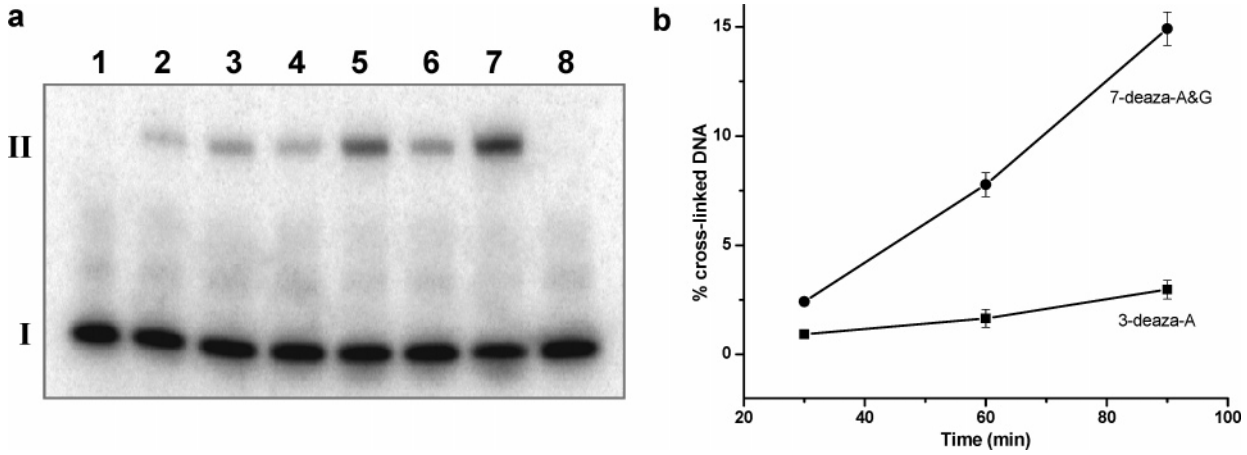


FIGURE 2: Photo-cross-linking reactions between platinated probes and hTBP. (a) Phosphorimage of a denaturing polyacrylamide gel. Bands I and II correspond to the non-cross-linked and photo-cross-linked probes, respectively. Lanes: (1) probe 4 without TBP, 90 min; (2) probe 4 + TBP, 30 min; (3) probe 3 + TBP, 30 min; (4) probe 4 + TBP, 60 min; (5) probe 3 + TBP, 60 min; (6) probe 4 + TBP, 90 min; (7) probe 3 + TBP, 90 min; (8) probe 3 + TBP, 90 min, NaCN. All samples were irradiated at 312 nm for the indicated time intervals. (b) Time dependence of photo-cross-link formation monitored by integrating intensities of band II. Plotted data are averages of three independent experiments, and error bars represent ±1 standard deviation.

reduced but not completely abolished (Figure 1c). Photosubstitution of a platinum-bound ligand by a suitably positioned amino acid residue of bound TBP, we argued, might then induce a metal-mediated ternary complex. As TBP makes contacts exclusively with the minor groove, evidence of photo-cross-link formation would support the predicted molecular mechanism of TBP binding inhibition.

A cross-linking assay was performed on the complexes formed between TBP and probes 3 or 4 platinated with 0.5 molar equiv of PT-ACRAMTU. Sufficient TBP was added to platinated probes to achieve 60% bound duplex based on K_d determinations for each probe (Figure S4). PT-ACRAMTU induces protein−DNA cross-links upon irradiation with 312 nm light as evidenced by the appearance of a high-molecular-weight band on the denaturing PAGE gel (Figure 2a). Addition of sodium cyanide to the mixture reverses the cross-link, confirming that platinum is the mediator of ternary complex formation (Figure 2a). Characteristically, the high-molecular-weight complex is not observed in the absence of light or in the absence of TBP. At each irradiation time, more cross-linked product is observed for the TBP−probe 3 complex than for the TBP−probe 4 complex. This is consistent with the expectation that probe 3 contains primarily minor groove adducts, thus favorably positioning platinum for UV-induced cross-linking. In contrast, probe 4 lacks minor groove adducts and forms cross-links less efficiently than probe 3 (Figure 2b).

DISCUSSION

We previously proposed (*29*) that the cytotoxic effect of the platinum intercalator conjugate PT-ACRAMTU may be generated by multiple mechanisms, including topoisomerase poisoning. PT-ACRAMTU promiscuously damages G and A sites in random-sequence DNA, and adducts are formed at both major and minor groove positions. Given that RNA polymerases bind DNA in the minor groove, we hypothesized that transcription inhibition might be one of the cytotoxic implications of DNA damage. Indeed, transcription catalyzed by T7 RNA polymerase was significantly impaired even at low drug concentrations (*32*). To dissect the defect responsible for transcription inhibition, we looked here to the eukaryotic RNA polymerase II, which recognizes its promoter through TBP−TATA box binding.

The TATA box consensus sequence, TATA(T/A)A(T/A)N (where "N" is any base), is an intrinsically deformable nucleotide tract (*41*). The saddle-shaped TBP associates with its A/T-rich promoter through the minor groove and induces a sharp bend in the DNA duplex leading to compression of the major groove (*41*). This distinct bending is a consequence of the molecular mechanism of TBP binding, in which partial insertion into the base stack of four Phe residues from the minor groove produces large roll angles (∼50°) at the outermost base pair steps of the TATA box. The complex thus formed is stabilized primarily by van der Waals interactions involving Val, Leu, and Ile residues of TBP and the minor groove walls, as well as by sequence-independent hydrogen bonding between charged and polar residues of TBP and the phosphodiester backbone (*41*). The lack of base-specific contacts observed in the crystal structures provides a rationale for the observed variation in TATA box sequences. A limited number of hydrogen bonds are also observed at the bottom of the minor groove between A-N3 and T-O6 and TBP Thr and Asn residues (*41*). While high-resolution structural information for the adenine minor groove adduct of PT-ACRAMTU is not yet available, the binding mode of the G-N7 adduct has recently been determined by NMR (*42*). On the basis of this structure, we predict that the minor groove adduct involves platination of A-N3 and intercalation of the acridine chromophore into the base stack on the 5′ face of the modified nucleobase, placing acridine between T and A. Such bulky adducts would cause inhibition of TBP binding by disrupting critical groove contacts. In the crystal structure of hTBP bound to the adenovirus E4 promoter sequence, for instance, Asn-163 and Asn-253 are involved in hydrogen bonding with A-N3 at the central AT step (*41*). If this A-N3 H-bond acceptor was instead modified with platinum, TBP binding would no doubt be significantly altered. Another mechanism of inhibition may involve direct competition between acridine and Phe intercalation at the terminal TA steps of the TATA box.

Partial intercalation and unstacking of the TA base pairs by TBP's Phe residues are a prerequisite for protein-induced DNA bending and complex formation. Intercalated acridine may not only prevent this crucial interaction but also stabilize an unbent, rodlike DNA conformation, as seen in the solution structure of the G-N7 monoadduct (*42*). Indeed, the 8−9° increase in duplex melting temperature upon platination (Figure S1) indicates that the normally flexible TA tract is stabilized, shifting the equilibrium away from TBP binding and the bent conformation. Furthermore, the increase in melting temperature also confirms the monofunctional coordinative-intercalative mode of PT-ACRAMTU binding, as monoplatination without intercalation has been shown to decrease thermal stability of DNA duplexes (*47*).

A critical structural difference also emerged between the effects of the major groove adducts formed by PT-ACRAMTU and the cisplatin analogue, enplatin. Under conditions where the formation of minor groove adducts by PT-ACRAMTU is prohibited by eliminating A-N3 donor sites, TBP binding is not affected (probe 4, Figure 1c). Thus, it can be concluded that the PT-ACRAMTU major groove adducts formed have no effect on TBP binding. In contrast, the 1,2 intrastrand cross-links formed between adjacent G-N7 positions by classical enplatin enhance TBP binding by 1.5-fold when both flanking GG sites are cross-linked (Figure 1c, at a drug:probe ratio of 2:1). Such enhancement is likely due to TBP recognizing the "pre-bent" DNA structure, as TBP binds cisplatin-modified DNA even in the absence of a TATA box sequence (*20*). Shape-specific binding has also been attributed to the HMG proteins, which strongly associate with GG cross-linked DNA.

To confirm that minor groove adducts formed by PT-ACRAMTU are directly involved in the inhibition of TBP binding, we cross-linked platinated DNA to TBP. While the exact mechanism of cross-link formation is still unknown, we speculate that photodissociation of the en-NH_2 group (*48*) trans to acridine sulfur may activate the metal for substitution reactions without labilizing the Pt−DNA bond. Suitable nucleophilic amino acid side chains of TBP in close proximity to the adduct may then form a coordinative bond to the metal center. Candidate amino acids include the Thr and Asn residues shown in the TBP−TATA box crystal structure to contact the DNA minor groove (*41*). Formation of the platinum-mediated ternary DNA−drug−protein complex demonstrates that the damaged TATA box is recognized by TBP, although with 25% reduced affinity (Figure 1c). Thus, at low degrees of platination the TBP and drug apparently co-occupy the TATA box. This may result in an improper orientation of TBP relative to the DNA promoter, ultimately leading to faulty assembly of the RNA polymerase II holoenzyme complex and impaired transcription (*49*).

PT-ACRAMTU is the first platinum-based agent to directly interfere with TBP binding, a mechanism previously known for covalent and noncovalent intercalators and groove binders (*50*, *51*). The inhibition of TBP binding is due to damage at A-N3, as evidenced by our use of artificial nucleobases to enhance minor groove adduct formation and block minor groove H-bond acceptors. Given that A-N3 damage accounts for only 5−10% of the adducts formed by this drug, simple blocking of TBP binding is not expected to be a major cause of cytotoxicity. Nevertheless, we argue that such adducts, if formed at a high frequency in genomic DNA, may contribute to the cytotoxicity of this drug by interfering with transcription. In native DNA, with all major and minor groove sites available for adduct formation, treatment with PT-ACRAMTU results in inhibition of TBP binding by approximately 11%. Furthermore, we expect that transcription inhibition arises from the cumulative effects of DNA damage. In addition to blocking TBP binding, adduct formation may produce a TBP−promoter complex that is an ineffective platform for assembly of other transcription factors, such as TFIIA and TFIIB. Additionally, minor groove adducts formed along the template sequence may prevent elongation of the nascent RNA. Future studies, therefore, will be concerned with the design of TATA sequence and minor groove specific agents derived from PT-ACRAMTU and their effects on RNA polymerase II transcription activity.

SUPPORTING INFORMATION AVAILABLE

Figures showing biophysical characterization of DNA probes (UV melting curves, CD spectra), MALDI-TOF mass spectra of platinated probes, autoradiograms of EMSA experiments, graphical determination of dissociation constants, and autoradiograms of thermal and photo-cross-linking experiments. This material is available free of charge via the Internet at http://www.pubs.acs.org.

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