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Consequences of Nucleic Acid Conformation on the Binding of a Trinuclear Platinum Drug†

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ABSTRACT: BBR3464, a charged trinuclear platinum compound, is the first representative of a new class of anticancer drugs to enter phase I clinical trials. The structure of BBR3464 is characterized by two [*trans*-$PtCl(NH_3)_2$] units linked by a tetraamine [*trans*-$Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2$] unit. The +4 charge of BBR3464 and the separation of the platinating units indicate that the mode of DNA binding will be distinctly different from those of classical mononuclear drugs such as cisplatin, *cis*-$[PtCl_2(NH_3)_2]$. The reaction of BBR3464 with three different nucleic acid conformations was assessed by gel electrophoresis. Comparison of single-stranded DNA, RNA, and double-stranded DNA indicated that the reaction of BBR3464 with single-stranded DNA and RNA was faster than that with duplex DNA, and produced more drug−DNA and drug−RNA adducts. Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry was used to further characterize the binding modes of BBR3464 with the DNA substrates. BBR3464 binding to different nucleic acid conformations raises the possibility that the adducts of single-stranded DNA and RNA may play a role in the different antitumor efficacies of this novel drug as compared with cisplatin.

BBR3464[1] (Figure 1), a novel trinuclear platinum compound that entered phase I clinical trials in June 1998, represents a new class of antitumor drugs that is not based on the classical mononuclear cisplatin structure, *cis*-$[PtCl_2(NH_3)_2]$. The structure of BBR3464 is characterized by two [*trans*-$PtCl(NH_3)_2$] units linked by a tetraamine [*trans*-$Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2$] unit and is based on the dinuclear concept developed in our laboratories (*1*−*4*). BBR3464 exhibits more potent antitumor activity than

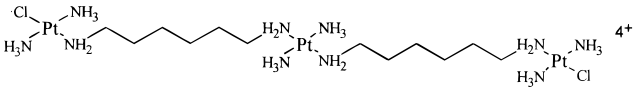
oligo1: 5′-CAGCGTGCGCCATCCTTCCC-3′

oligo2: 5′-GGGAAGGATGGCGCACGCTG-3′

oligo3: 5′-CAGCGUGCGCCAUCCUUCCC-3′

oligo4: 5′-GGGAAGGAUGGCGCACGCUG-3′

duplex: 5′-CAGCGTGCGCCATCCTTCCC-3′
3′-GTCGCACGCGGTAGGAAGGG-5′



BBR3464

FIGURE 1: Sequences of DNA and RNA and the structure of BBR3464.

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cisplatin. The profile of antitumor activity is also distinctly different (*5*). The drug demonstrates activity in human tumor (e.g., ovarian) xenografts resistant to cisplatin and alkylating agents, and high activity in a broad spectrum of human tumors commonly insensitive to chemotherapeutic intervention (e.g., non-small-cell lung, gastric) and characterized as p53 mutant (*6*, *39*).

It is generally accepted that DNA is the main pharmacological target of platinum(II) complexes. A plausible explanation, then, is that the differential antitumor activity of BBR3464 as compared to that of cisplatin is related to its mode of DNA binding (*4*). The modes of DNA binding of BBR3464 are unlike that of any other clinically used DNA-damaging agent. The structural features to consider for DNA binding are (i) the presence of charge and hydrogen-bonding along the backbone of the molecule, contributed by the central tetraamine platinum coordination unit, which does not bind covalently to the oligonucleotide; and (ii) the bifunctional binding effected by substitution of both Pt−Cl bonds in the molecule. Recent findings in our laboratory have shown that BBR3464 is capable of bifunctional binding through both long-range interstrand and intrastrand cross-links on duplex DNA (*7*). On the basis of molecular modeling and the crystal structure of BBR3464 (*8*), possible drug−DNA interactions extend from 1,2- to at least 1,6-interstrand or intrastrand cross-links.

In this paper, we present the results of a model study on the formation of BBR3464−nucleic acid adducts using three different oligonucleotide substrates: single-stranded DNA, single-stranded RNA, and double-stranded DNA. BBR3464 preferentially binds short single-stranded DNA and RNA and leads to more drug adducts, as compared to double-stranded DNA. Further, we demonstrate that electrospray ionization (*9*) coupled with Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) (*10*, *11*) is a powerful method for characterizing the modes of binding of the drug to an oligonucleotide. Direct visualization of drug−DNA adducts by ESI-FTICR-MS showed that both bifunctional and monofunctional substitution occurs on single-stranded DNA, even after reaction for only 1 h. BBR3464 binding to different nucleic acid conformations raises the possibility that the adducts of single-stranded DNA and RNA, as well as the unique adducts with double-stranded DNA, may play a role in the different antitumor efficacies as compared with those of cisplatin.

EXPERIMENTAL PROCEDURES

Starting Materials and Physical Methods. UV−visible spectra were collected on a Hewlett-Packard 8452A diode array spectrometer, and nucleic acid concentrations were calculated by measuring the UV absorbance at 260 nm.

Synthetic oligodeoxyribonucleotides were purchased from Operon Technologies (Alameda, CA), and oligoribonucleotides (which are the comparable RNA sequences, i.e., U replacing T) were purchased from Oligos Etc. (Bethel, ME). All oligonucleotides were purified on 20% polyacrylamide gels under denaturing conditions prior to use. To make duplex DNA, the complementary oligonucleotides were mixed at a 1:1 molar ratio, heated at 90 °C for 5 min, and slowly cooled to room temperature to allow annealing of the two strands. The duplexes were purified by electrophoresis on native 10% polyacrylamide gels and eluted by standard methods (*12*). The DNA was precipitated and resuspended in 10 mM Tris-HCl and 1 mM EDTA (pH 8) (TE) and stored at −20 °C. The single-stranded oligonucleotides and the duplex were 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP.

BBR3464 was a gift and was synthesized at Boehringer Mannheim Italia, now Novuspharma Spa (Monza, Italy), as described previously (*8*). Stock solutions of the drug (5−500 μM) were prepared in doubly distilled H_2O and stored at −20 °C.

Platination Reactions. All platination reactions were performed in H_2O at 37 °C. The nucleic acid (100 nM single-stranded DNA or RNA and 100 nM duplex) was mixed with 1 molar equiv of BBR3464 and the mixture incubated at 37 °C. Aliquots of the reaction mixture were withdrawn at various times and were ethanol precipitated to both terminate the reaction and remove any unbound drug. Samples were resuspended in TE and divided into two sets. One set was analyzed by gel electrophoresis under denaturing conditions (16% polyacrylamide/8 M urea at 50 °C), and the other set was analyzed by gel electrophoresis under native conditions (10% polyacrylamide). The amount of free and adducted DNA or RNA was determined with a Molecular Dynamics PhosphorImager using ImageQuant software, and the data were averaged over at least five independent experiments.

Mass Spectrometry. Preparation of drug−DNA complexes was performed in one of two ways. First, DNA (50 nmol of single-stranded DNA or 50 nmol of duplex DNA) was simply mixed with 1 molar equiv of drug in water and the mixture incubated at 37 °C for 1 h, followed by ethanol precipitation to terminate the reaction. Second, the identical reaction was carried out, for oligo1 only, and the drug−oligo1 complexes were separated by electrophoresis on 20% polyacrylamide gels under denaturing conditions. The drug−DNA complexes were visualized by ethidium bromide staining; two of the bands (bands I and II) were excised from the gel, and the drug−DNA adducts were eluted by standard methods (*12*). All samples, independent of preparation, were then subjected to microdialysis which is described in detail elsewhere (*13*). Briefly, the samples were infused at a flow rate of 2 μL/min into the conical microdialysis membrane against a counter-current buffer of 10 mM ammonium acetate with a gravity-induced flow limited to 1.3 mL/min using a screw clamp. Concentrations of the drug−DNA adducts were calculated using Beer's Law and on-line UV absorbance readings using a microflow cell at the exit of the microdialysis unit, and were determined to be 116 μM which was then diluted to 10 or 20 μM in the electrospray buffer (vide infra) (*13*).

The dialyzed reaction mixtures were electrosprayed from a 50 μm fused-silica capillary pulled to a fine tip and remotely coupled to a potential of ca. −2100 V with a flow rate of 300 nL/min (*14*). The electrospray tip was positioned directly in front of the ESI source (Analytica of Branford,

[1] Abbreviations: BBR3464, [*trans*-diammino-bis{*trans*-chlorodiammino-μ-(1,6-hexanediamino)platinum(II)}platinum(II)tetranitrate]; cisplatin, *cis*-diamminedichloroplatinum(II) {*cis*-[$PtCl_2(NH_3)_2$]}; ESI-FTICR-MS, electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; EDTA, ethylenediaminetetraacetic acid; M_O, molecular ion of an unadducted oligodeoxynucleotide; M_M, molecular ion of a monofunctional adduct of BBR3464 complexed to the oligodeoxynucleotide; M_B, molecular ion of a bifunctional adduct of BBR3464 complexed to the oligodeoxynucleotide.

Branford, CT), modified to accept a heated metal capillary (*15*) that was held at 200 °C for all the work described here. The mass spectrometer was an IonSpec (Irvine, CA) 4.7 T Fourier transform ion cyclotron resonance mass spectrometer as previously described (*13*). All spectra were obtained using a broad-band pulse sequence and a preamplifier gain of 1×, without a window function, and 512K data points were acquired at a 500 kHz ADC rate. The addition of organic solvents and additives for electrospray resulted in the following solution compositions: 50%/50% 2-propanol/10 mM ammonium acetate. All electrospray reagents were purchased from Sigma at the highest purity available and used as received. Theoretical most-abundant isotope mass determinations were made using ICR-2LS (*16*) which incorporates the fast Fourier transform algorithm introduced by Rockwood et al. (*17*).

RESULTS

Pt−DNA and Pt−RNA Adducts. Figure 1 shows the oligonucleotides and drug used in this study. The sequences correspond to a guanine-rich region of the gene for BCL-2, a suppressor of apoptosis, and contain a population of potential drug-binding sites. To explore the preferential reactivity of BBR3464 with single-stranded DNA and RNA as compared to that with duplex DNA, complex formation was monitored for 1:1 molar ratios of drug to nucleic acid over time at 37 °C (Figure 2A). Treatment of the three different nucleic acid conformations with BBR3464 gave a complex mixture of products, as indicated by the appearance of multiple slower migrating bands. Remarkably, the BBR3464 reactions with all single-stranded substrates gave a population of complexes within 30 s. After only 5 min, approximately 30% of oligo1/oligo3 and 40% of oligo2/oligo4 are covalently complexed with the drug, as compared to only 10% of the duplex. The amount of unbound DNA and RNA remaining as a function of time (Figure 2B) clearly shows that the single-stranded oligonucleotides react faster with BBR3464 than the duplex, and produce a greater percentage of drug complexes. The single-stranded DNA reactions plateau with approximately 80% of the DNA complexed; the RNA reactions plateau with approximately 95% of the RNA complexed, while the double-stranded DNA reactions plateau with only 50% of the duplex DNA complexed. Note the duplex does not dissociate after binding of BBR3464, indicating that the covalent attachment of the drug does not significantly disrupt the hydrogen bonding in the helix (Figure 2C, lanes 18−26).

Characterization of Pt−DNA Adducts. Panels A and B of Figure 3 show the ESI-FTICR mass spectra of oligo1 and oligo2, respectively, reacted with 1 molar equiv of BBR3464 in H_2O for 1 h at 37 °C. The assignment of the unadducted oligodeoxynucleotide and characterization of the various binding modes of the trinuclear compound with the oligodeoxynucleotide were accomplished using accurate mass measurements. Mass calibration was accomplished using the first five isotopic peaks of the −6, −7, and −8 charge states of the free (unreacted) oligonucleotide (M_O) as the internal mass standard, which avoids space-charge effects (*18*) and afforded low to sub-parts per million mass accuracy. All masses are reported as the most abundant isotopic mass for the neutral molecules. Due to the large isotopic shift (∼18 Da) introduced as a result of the three Pt atoms in the molecule, the monoisotopic mass, which is less than 0.0005% of the most abundant isotopic peak, was not detected. A bifunctional adducted DNA ion exhibiting the same charge state in the gas phase as a monofunctional adducted ion is differentiated by a displaced Cl, which occurs upon the covalent attachment of BBR3464 to the DNA, and one additional site of deprotonation on the phosphodiester backbone to account for the increased charge on the Pt. Bifunctional (M_B) and monofunctional (M_M) adducts are observed for oligo1 (Figure 3A), whereas the bifunctional adduct was the only adduct observed for oligo2 (Figure 3B). Preliminary results for the BBR3464−duplex DNA reaction indicate the presence of only bifunctional adducts (data not shown). Further characterization of the drug−duplex DNA and drug−RNA complexes is being carried out to determine the specific nature of these adducts. Attempts to collisionally activate and dissociate the monofunctional and bifunctional

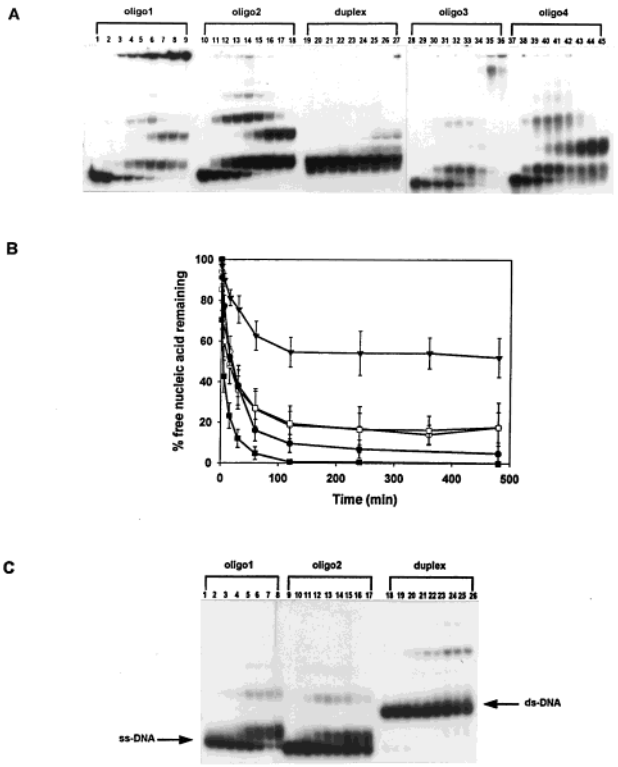

FIGURE 2: Reactivity of single-stranded oligonucleotides and duplex DNA with BBR3464. (A) Reaction mixtures containing oligo1 (lanes 1−9), oligo2 (lanes 10−18), duplex (lanes 19−27), oligo3 (lanes 28−36), and oligo4 (lanes 37−45) were mixed with 1 molar equiv of BBR3464, and aliquots were removed at 0.5, 5, 15, 30, 60, 120, 240, and 480 min, respectively, and assayed on 16% denaturing polyacrylamide gels. Lanes 1, 10, 19, 28, and 37 contained the indicated nucleic acid substrate with no drug treatment. (B) The amount of unbound DNA and RNA remaining as a function of time: oligo1 (■), oligo2 (□), duplex (○), oligo3 (●), and oligo4 (▼). The data are the average of at least five independent experiments. (C) Reaction mixtures containing DNA and 1 molar equiv of BBR3464 assayed on 10% native polyacrylamide gels. Lane 1 contained oligo1 with no drug treatment. Lanes 2−8 contained oligo1 treated with BBR3464 after 0.5, 5, 15, 30, 60, 120, and 480 min, respectively. Lane 9 contained oligo2 with no drug treatment. Lanes 10−17 contained oligo2 treated with BBR3464 after 0.5, 5, 15, 30, 60, 120, 240, and 480 min, respectively. Lane 18 contained duplex with no drug treatment. Lanes 19−26 contained duplex treated with BBR3464 after 0.5, 5, 15, 30, 60, 120, 240, and 480 min, respectively.

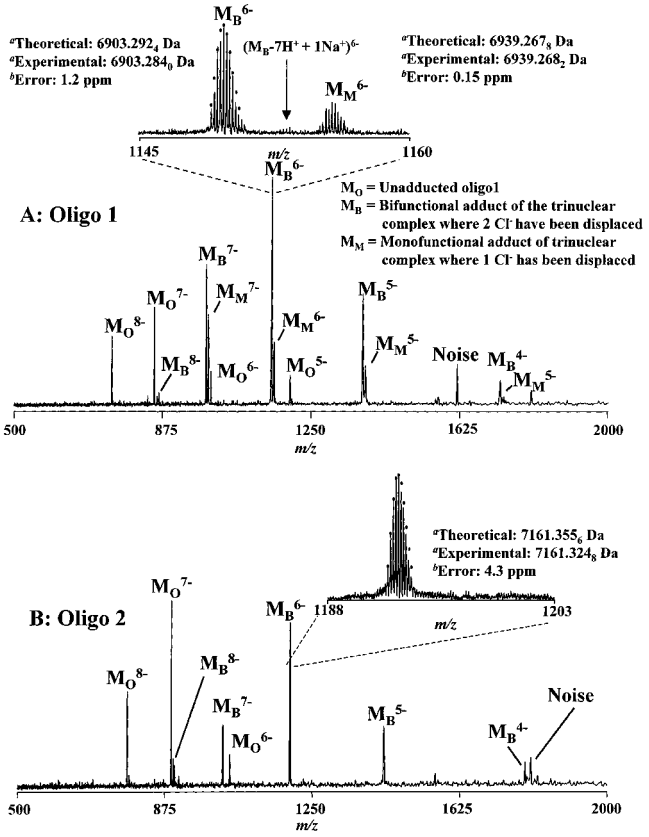

FIGURE 3: ESI-FTICR mass spectra of oligo1 (A) and oligo2 (B) reacted with 1 molar equiv of BBR3464 for 1 h at 37 °C. The theoretical isotopic distribution, indicated by the circles, agrees well with the experimental isotopic distribution. [a]The most abundant isotopic mass for the neutral molecules is reported. Due to the large isotopic shift (~18 Da) introduced as a result of three Pt atoms being in the molecule, the monoisotopic mass, which is less than 0.0005% of the most abundant isotopic peak, was not detected. [b]Internal calibration was achieved using the first five isotopic peaks of the −6, −7, and −8 charge states of M_O.

species in the gas phase using sustained off-resonance irradiation (*19*) resulted in product ions indicative of neutral base loss (i.e., no structural fragments).

The high mass resolution obtained by simply mixing BBR3464 and the oligonucleotide encouraged us to examine the chemical nature of the multiple products present in the gel studies. Figure 4 shows the ESI-FTICR-MS data obtained for two gel-isolated adducts of the BBR3464−oligo1 reaction (Figure 4A). Panels B and C of Figure 4 show the mass spectra of the isolated species present in bands I and II, respectively. The dominant species in both cases are free oligonucleotide (M_O) and a bifunctional drug adduct (M_B). Conclusions concerning the ratio of free oligonucleotide to bifunctional adduct are not possible since the steps involved in sample preparation (extraction, precipitation, and microdialysis) can alter the relative recoveries at each stage of the separation. Interestingly, additional peaks corresponding to decomposition of the drug−DNA adduct were present in the spectra for both gel isolates. Species labeled α, *β*, and *γ* observed in the ESI-FTICR mass spectra correspond to M_O + $Pt(NH_3)_2$, M_B − $Pt(NH_3)_2$, and M_B + $Pt(NH_3)_2$, respectively. These features were not observed in the ESI-FTICR mass spectrum of the reaction mixture from aqueous solution (see Figure 3A), suggesting that the treatment of the drug−

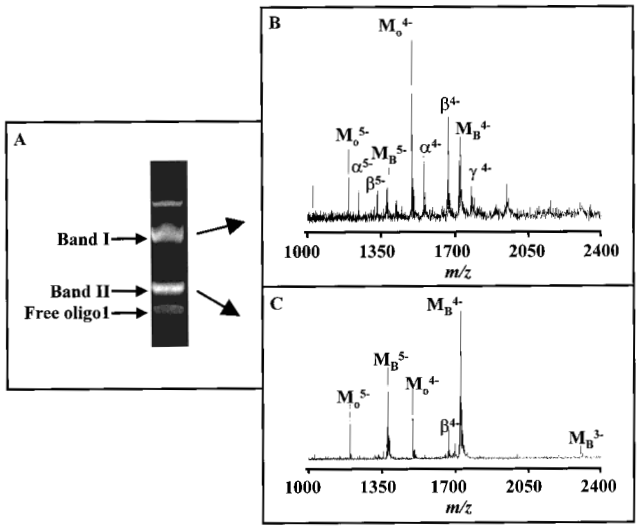

FIGURE 4: ESI-FTICR data obtained for two gel-isolated adducts of the BBR3464−oligo1 reaction. (A) Reaction of oligo1 with 1 molar equiv of BBR3464 for 1 h at 37 °C assayed on a 20% denaturing polyacrylamide gel and visualized by ethidium bromide staining. (B) ESI-FTICR mass spectrum of band I. (C) ESI-FTICR mass spectrum of band II. Species labeled α, *β*, and *γ* observed in the ESI-FTICR mass spectra correspond to M_O + $Pt(NH_3)_2$, M_B − $Pt(NH_3)_2$, and M_B + $Pt(NH_3)_2$, respectively.

DNA adducts during gel isolation of the complexes is responsible.

DISCUSSION

Sequencing studies on plasmid DNA have confirmed the preferential binding of BBR3464 to guanosine residues (*6*, *39*). The overall binding of BBR3464 to single-stranded or double-stranded nucleic acids may be considered as a three-step process: rapid initial electrostatic binding followed by covalent attachment of the drug by sequential displacement of the leaving group (Cl or H_2O) to the N7 position of guanosines, giving first monofunctional and subsequently bifunctional adducts. Chemical studies to date indicate that substitution occurs through the initial formation of aquo species, similar to the situation for cisplatin (N. Farrell, unpublished data). Hydrolysis of the Pt−Cl bonds will give aquo products with a total charge of +5 (one Cl displaced) or even +6 (two Cl displaced). Previous studies with calf thymus DNA incubated with BBR3464 showed rapid binding of the drug with a $t_{1/2}$ of 40 min, and quantitative binding of BBR3464 after 6 h (*7*, *20*). Rapid binding of BBR3464 with the short oligonucleotides is also evident; the percentage of free nucleic acid substrate remaining in the reaction, for all species, remains constant after 120 min, although the population of complexes continues to change over time.

The sequences chosen for this kinetic study have multiple potential binding sites: four G's in oligo1 and oligo3 and ten G's with four GG steps in oligo2 and oligo4. Intuitively, we expected the two sets of single-stranded oligonucleotides (oligo1/oligo3 and oligo2/oligo4) to react differently to platination by BBR3464, on the basis of not only the different number of potential binding sites but also the different sequence context of those sites, with the reasonable assumption of only guanosine N7 attachment (*7*). The reaction of the nucleic acid substrates with BBR3464 can be dissected into two components: the rate of disappearance of the free

nucleic acid and the population of complexes formed from the drug reaction. The reactivity of all four single-stranded nucleic acid substrates is significantly better than that of double-stranded DNA, based on the amount of free nucleic acid remaining as a function of time (Figure 2B). The two single-stranded DNA substrates (oligo1 and oligo2) exhibit nearly identical profiles for the disappearance of free oligonucleotide, and are somewhat less reactive than their RNA counterparts (oligo3 and oligo4), which exhibit some slight variation in reactivity between the two different sequences. These data suggest that the initial rapid electrostatic interaction of the +4 dichloro species (or +5 and +6 in the case of the monoaquo and diaquo species, respectively) of BBR3464 presumably dominates in determining the rate of binding to single-stranded oligonucleotides.

However, on the basis of electrostatics alone, we would predict the positively charged BBR3464 to preferentially bind to duplex DNA (double the negative charge on the backbone) as compared to single-stranded DNA or RNA. The remarkable reactivity of BBR3464 with single-stranded DNA and RNA, and no reactivity of duplex DNA, within 30 s suggests the possibility that the conformation of the double-stranded DNA itself is responsible for the difference in the observed reactivities. Since the double helix is conformationally restrained relative to single-stranded DNA and RNA, the linear BBR3464 cation must adopt the correct orientation for maximal electrostatic interaction and subsequent covalent bond formation. Alternatively, if the mechanism of drug−DNA binding involves unwinding of the helix for optimal positioning of the Pt−N7 bond (structural changes necessary for bifunctional binding), then single-stranded DNA and RNA can be considered already "unwound", resulting in a lower energy barrier for bifunctional binding. The similar reactivity profiles for the rate of disappearance of the single-stranded DNA molecules and the slight preference of BBR3464 for single-stranded RNA may reflect the highly polymorphic nature of single-stranded conformations. Presumably, the two DNA single-stranded molecules adopt similar geometries in solution, while the two RNA molecules may have increased conformational variability; e.g., oligo4 has the potential to form a relatively stable stem−loop structure. It is important to note that the kinetic studies of our model system measure only the rate of disappearance of free oligonucleotide and do not distinguish between monofunctional and bifunctional adduct formation as the final product. Thus, the enhancement of reactivity between single-stranded nucleic acids as compared to that of duplex DNA most likely reflects their conformational differences, since it cannot be interpreted to reflect the different nature or rate of formation of the covalent adducts formed among the three different nucleic acid conformations.

While nucleic acid conformation plays an important role in the initial binding of BBR3464, the band migration patterns of the different substrates suggest that the drug does indeed form different covalent adducts based on sequence context. Characterization of DNA adducts has previously been achieved with ESI-MS (*21*−*25*); however, the inherent resolution and mass accuracy of an ESI-FTICR approach allowed the complex isotopic distribution of the triplatinum compound to be resolved, thus affording accurate mass measurements using internal mass calibration. The ESI-FTICR-MS data obtained for the 1 h reaction of BBR3464 with the three different DNA substrates indicate that oligo1 forms both monofunctional and bifunctional adducts, while oligo2 forms only bifunctional adducts. Preliminary data for the double-stranded DNA indicate only bifunctional adducts. Further analyses of the drug−DNA reaction by mass spectrometry, using gel-isolated complexes of two of the species present in the BBR3464−oligo1 reaction, reveal a more complex picture of the reaction. In this detailed example of oligo1, it would be intuitively tempting to assign band II to a monofunctional species and band I to a bifunctional species, on the basis of charge considerations of the drug−DNA complexes; the overall charge of the DNA with one monofunctional drug adduct is −14 (−19 + 5), and with one bifunctional drug adduct is −13 (−19 + 6) (Figure 4A). The mass spectrometry data obtained for the BBR3464−oligo1 reaction indicate both gel-isolated complexes contain bifunctional adducts, despite the difference in the electrophoretic mobility of the two bands. Bifunctional binding using the four guanosine residues of oligo1 can produce a range of 1,3-, 1,5-, and 1,7-intrastrand cross-links, which also differ among themselves depending on sequence. A 1,5-intrastrand cross-link, for example, could be formed between G_3 and G_7 or between G_5 and G_9. Presumably, the difference in migration results from these different sites of bifunctional binding. Differences in migration patterns of interstrand cross-linked DNA have previously been ascribed to similar phenomena (*26*). In agreement, recent studies on the alkylation and cross-linking of DNA by mitomycin C clearly demonstrated that the position of the cross-link within the DNA duplex affected the electrophoretic mobility of the complex in denaturing gels (*27*). While this does not affect the arguments presented here, due caution should be taken in assigning gel bands to specific chemical structures.

The differential reactivity of BBR3464 with respect to different nucleic acid conformations may have profound biological consequences. For DNA-damaging drugs such as platinum and alkylating agents, conformational changes upon binding to double-stranded DNA are usually invoked to explain their mechanism of action. The altered DNA helical structure potentially affects the recognition of the DNA by cellular proteins, the basis for the antitumor effects of the drug. Recent evidence with cisplatin−DNA lesions suggests the 1,2-intrastrand cross-link may block DNA replication and RNA transcription (*28*) or alter recognition of DNA repair proteins (*29*−*31*), or alternatively, the lesions may recruit proteins containing the HMG (high-mobility group) box binding motif away from their cognate binding sites (*32*−*35*). The clinical development of BBR3464 arose through systematic drug development efforts having a hypothesis that novel DNA-binding modes will produce antitumor activity complementary to that of cisplatin (*1*−*4*). As a corollary of our current understanding of the cisplatin mechanism, structurally unique DNA-binding modes would mediate their biological consequences through alternative protein signaling pathways.

On the basis of the observation that BBR3464 is more reactive with respect to single-stranded DNA than duplex DNA, adducts on single-stranded conformations may also be an effective mechanism for disrupting cellular processes. Rapid access to the genome could be enhanced by the greatly enhanced "DNA-affinic" nature of BBR3464 (essentially a highly charged polyamine capable of covalent binding) as

compared to that of small neutral molecules. Single-stranded DNA is present during transcription, replication, recombination, and repair, and is recognized by various single-stranded DNA binding proteins. Inhibition of these additional cellular machineries may contribute to the more potent antitumor activity of BBR3464 as compared to cisplatin. The ability of BBR3464 to form intrastrand cross-links on single-stranded DNA also suggests an important mechanism for binding of the drug to duplex DNA. Quantitation of BBR3464 cross-linking to duplex DNA in a linearized plasmid, under conditions where all the drug is complexed, indicated approximately 20% of the drug−DNA adducts were interstrand cross-links, significantly higher than the value of 6% for cisplatin (*7*). The confirmation by mass spectrometry of rapid bifunctional covalent attachment of BBR3464 to single-stranded oligonucleotide substrates in this paper indicates that the remaining (predominant) lesions in duplex DNA are indeed intrastrand cross-links.

The enhanced binding of BBR3464 to single-stranded RNA substrates suggests additional pathways for disrupting cellular function. The rapid initial binding of BBR3464 to the oligoribonucleotides in this study suggests that electrostatic interactions between BBR3464 and RNA may be an important contribution to molecular recognition. This may be analogous to the targeting of RNA by aminoglycoside antibiotics, with the caveat that the RNA targets have distinct secondary structures. These polycationic amino-modified oligosaccharides are able to interfere with ribosomal protein synthesis, viral replication, ribozyme function, and intron splicing, presumably through electrostatic interactions (*36*).

In tissue culture and in vivo experiments, cell lines with mutant p53 are exquisitely sensitive to BBR3464 (*6*, *39*). The p53 tumor suppressor protein is involved in the regulation of several cellular responses, including cell cycle control, DNA repair, and apoptosis. Recent evidence with various DNA-damaging agents has indicated that the mechanism of action of several drugs involves a complex balance between p53-dependent and p53-independent pathways (*37*, *38*). A plausible explanation for this sensitivity, then, is that the novel DNA-binding modes of BBR3464 are not recognized by p53 and so repair and/or apoptotic pathways are not induced. Ongoing studies of the relationship of single-stranded and double-stranded DNA damage by BBR3464 represent powerful tools for understanding how BBR3464-induced lesions may contribute to cytotoxicity through p53-dependent and/or p53-independent apoptotic pathways, thus elucidating molecular mechanisms (specific DNA binding modes) for altering cellular signaling pathways.

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