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Effect of Ribonucleotide Substitution on Nucleic Acid Bulge Recognition by Neocarzinostatin

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Abstract—Bulged RNA structures are not as good substrates for cleavage by the enediyne antibiotic neocarzinostatin chromophore in the general base-catalyzed reaction as are DNA bulges. In an effort to determine why this is so, we have systematically substituted ribonucleotide residues in a DNA bulged structure (CCGATGCG·CGCAGTTCGG) (cleaved residue is underlined) known to be an excellent substrate. It was found that ribonucleotide substitution at the bulge target site, as well as at other regions involving duplex formation had a small effect on the cleavage reaction, unless either of the two strands was entirely of the ribo form. By contrast, changing the A·T base pair on the 5′ side of the target nucleotide (T residue) to ribo A·U resulted in an 87% decrease in cleavage; in fact, conversion of the A alone to the ribo form caused a 68% loss in cleavage. This result can be understood from the recent solution structure of the complex formed between an analogue of the drug radical species and a bulged DNA (Stassinopoulos, A.; Ji, J.; Gao, X.; Goldberg, I. H. *Science* **1996**, *272*, 1943), since the 2′ hydroxyl group of the ribo A would be expected to clash sterically with the 7″-*O*-methyl moiety of the drug. Additional studies on substrate bulge-dependent drug product formation and protection against spontaneous drug degradation support the cleavage experiments, and imply that bulge-specific drug binding is required for efficient cleavage. © 1997 Elsevier Science Ltd.

Introduction

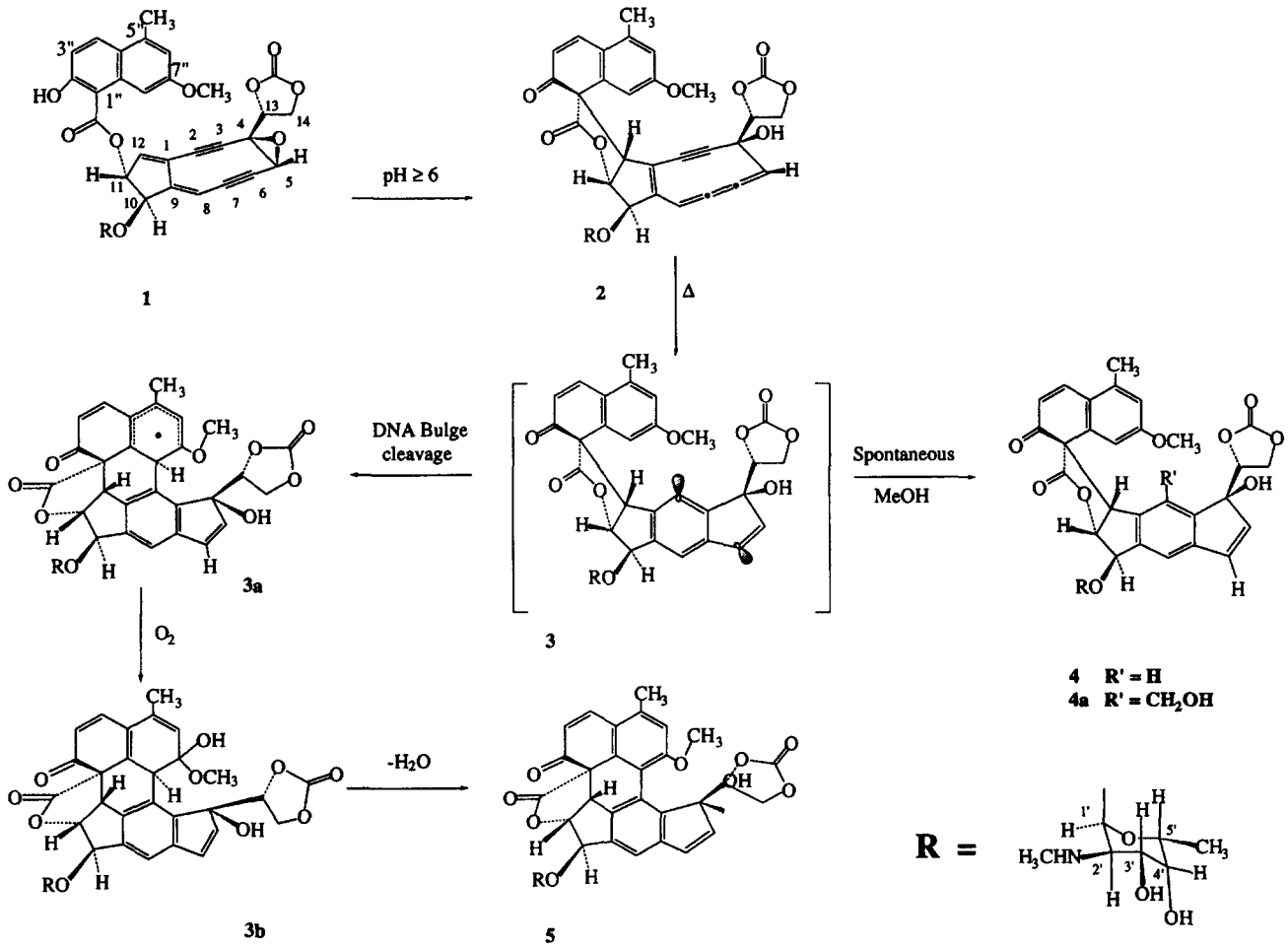
The antitumor drug neocarzinostatin chromophore (NCS chrom) normally requires a thiol activator to induce a variety of damages in duplex DNA.[1] An exception to this rule is the base-catalyzed, site-specific cleavage at a DNA bulge generated by the folding of single-stranded DNA or by the partial base pairing of two linear DNAs, one having extra bases.[2,3] The single-strand cleavage at the bulge is highly efficient, requires oxygen, and involves selective abstraction of the 5′-hydrogen at the target residue resulting in a PO_4 at the 3′-end and a nucleoside aldehyde at the 5′-end of the break. In addition, a unique drug product is generated in the bulged DNA reaction, indicating a novel mechanism of drug activation shown in Scheme 1.[2–7] The mechanism involves an intramolecular Michael addition of the phenol enolate at C1″ to C12 in NCS chrom (**1**) to give cumulene **2**, which undergoes a Bergman-type rearrangement to the biradical **3**. Biradical **3** is presumed to be the species involved in the abstraction of the 5′ hydrogen from the target nucleotide at the DNA bulge site and attack at C8″ of the naphthoate group. The novel drug product **5** results from biradical **3** through the proposed intermediacy of radical **3a** and compound **3b**. The base-catalyzed reaction in the absence of bulge DNA gives rise to the main spontaneous degradation products **4** and **4a**.[4,5]

While a wide range of DNA bulges, having varying sequences and structural features, proved excellent substrates for NCS chrom, RNA bulges were cleaved very poorly, although specifically.[3,6,7] For example, NCS chrom induced strand cleavage selectively at the bulge of the trans activation response region RNA (TAR RNA) of HIV type 1 but the efficiency of cleavage was very low.[6] This and similar weak cleavage obtained in other RNA substrates including tRNAs, presumably capable of forming bulges, suggested that the inherent structural differences between DNA and RNA play a critical role in the binding and/or positioning of the drug at the bulge site. In order to get further insight into the structure–activity relationship in the transition from DNA to RNA, deoxyribonucleotides in a proficient DNA substrate were sequentially replaced by ribonucleotides, and the resulting 'hybrid' duplexes were tested for cleavage by NCS chrom and for their ability to generate the bulge-specific drug product.

Results

Effect of ribosubstitution on site-specific cleavage

Figure 1 shows the highly efficient and selective site-specific cleavage by NCS chrom at the T residue in the bulge of a previously identified excellent DNA substrate made up of an 8-mer and a 10-mer (CCGATGCG·CGCAGTTCGG) (lane 2).[3] Substitution of the target T with ribo U decreases cleavage by only 25% (lane 3 and Table 1); the duplex having both bulge residues in the ribo form is cleaved somewhat less efficiently (lane 4), but still significantly. Of the two A–T base pairs in the Watson–Crick base-paired region, the one on the 5′-side of the target T at the bulge seems critical for drug activity, since changing this A–T to ribo A–U causes 87% loss of cleavage (lane 6). In fact, conversion of A alone to the ribo form is sufficient to effect significant changes in the microstructure of the duplex as



Scheme 1. Proposed mechanism for the intramolecular activation of NCS chrom in the absence or presence of bulged DNA under the influence of general-base catalysis.

evidenced by the loss of cleavage by 68% (lane 7). On the other hand, conversion of the A–T on the 3′-side of the target T to the ribo forms alters cleavage efficiency to a much lesser degree (lane 5). When all the residues of either strand are in the ribo form there is no significant cleavage (lanes 8 and 9, and Table 1).

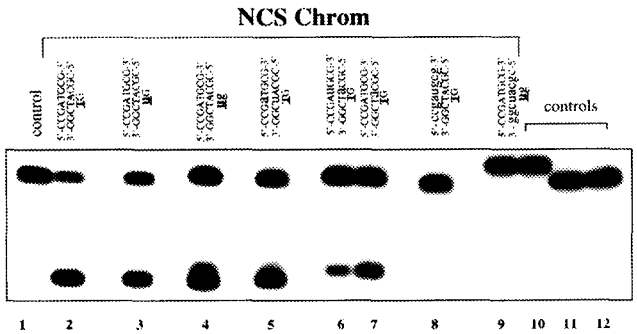


Figure 1. Effect of ribosubstitution on site-specific cleavage by NCS chrom. 5′-^{32}P-labeled 10-mer (44 μM), which had been annealed with the 8-mer, was treated with 44 μM NCS chrom as described in the Experimental section. Lower case letters denote ribo residues. Strand cleavage occurs at the underlined and bold residue. Lanes 1, 10, 11, and 12 contain no drug controls for the drug-treated samples in lanes 2, 9, 7, and 6, respectively.

In the next series of experiments, ribosubstitutions were made in three residues at either the 3′- or 5′- or both ends of the 10-mer and the 8-mer, and the duplexes of these in various combinations were tested as substrates for NCS. In substrates having three ribo residues only in one strand, either at the 5′- or the 3′-end, strand cleavage was nearly as good as that in the parent DNA duplex (data not shown). As shown in Table 2, duplexes where both strands have ribosubstitutions so as to form the three base-pair arm as DNA/RNA on both sides of the bulge, or RNA/RNA on only one side, were cleaved efficiently. The hybrid having all but the central core of six residues inclusive of the two-base bulge in the ribo form (duplex 6, Table 2) is still a reasonably good cleavage substrate at 44% efficiency.

Adduct formation

Site-specific cleavage by NCS chrom at the DNA bulge requires oxygen. Under anaerobic conditions strand cleavage is inhibited and a new product, presumably an adduct, that has a slower mobility on the gel than the starting material, is produced.[2,3] It was of interest to test whether ribo substitution affects the adduct formation. Figure 2 shows that under anaerobic conditions strand

Table 1. Effect of ribonucleotide substitution on cleavage

Substrate	Duplex	Cleavage (%)
1	5′-CCGATGCG 3′-GGCTACGC **T**G	100
2	5′-CCGATGCG 3′-GGCTACGC **u**G	75
3	5′-CCGATGCG 3′-GGCTACGC **u**g	60
4	5′-CCGaTGCG 3′-GGCuACGC **T**G	66
5	5′-CCGAuGCG 3′-GGCTaCGC **T**G	13
6	5′-CCGATGCG 3′-GGCTaCGC **T**G	32
7	5′-ccgaugcg 3′-GGCTACGC **T**G	4
8	5′-CCGATGCG 3′-ggcuacgc **u**g	2

The data are from the experiment in Figure 1. 100% represents 77% cleavage in the duplex composed of deoxyribonucleotides only. Lower case letters denote ribo residues. Cleavage occurs at the underlined and bold **T** or **u** residue.

Table 2. Effect of ribonucleotide substitution on cleavage

Substrate	Duplex	Cleavage (%)
1	5′-CCGATGCG 3′-GGCTACGC **T**G	100*
2	5′-CCGATgcg 3′-ggcTACGC **T**G	87
3	5′-ccgATGCG 3′-GGCTAcgc **T**G	54
4	5′-CCGATgcg 3′-GGCTAcgc **T**G	88
5	5′-ccgATGCG 3′-ggcTACGC **T**G	69
6	5′-ccgATgcg 3′-ggcTAcgc **T**G	44

5′-^{32}P-labeled 10 mer (22 μM) in the indicated duplex form was treated with an equimolar concentration of NCS chrom as described in Materials and Methods. *100% represents 65% cleavage. Lower case letters denote ribo residues. Cleavage occurs at the underlined and bold T residue in the bulge.

cleavage is inhibited in the DNA duplex 1, and in the hybrid duplex 2 of Table 2 with a concomitant increase in the presumed adduct(s) (lanes 3 and 5). Duplex 2 of Table 1, which has a U in place of the target T, also gave similar results (data not shown).

Chemistry of strand cleavage

The fact that strand cleavage by NCS chrom at the bulge in ribosubstituted-duplexes is spontaneous (without any additional treatment), and that the bands are coincident in their mobility on gels with that generated from the parent DNA duplex suggests that the chemistry of damage is not altered on ribosubstitution, and occurs by a mechanism involving the abstraction of 5′-hydrogen atom at the target residue, as established earlier.[2,3] The generation of the same drug product **5** with both DNA/DNA and DNA/RNA substrates (see below) further suggests the same mechanism of drug activation in both reactions.

Effect of ribosubstitution on formation of the drug product

A unique feature of the interaction between DNA bulge and NCS chrom is the generation of a bulge-dependent drug product (**5** in Scheme 1) concomitant with site-specific cleavage at the bulge.[2] Since generation of **5** requires binding of **2** (and **3**)[8] to the bulge substrate, ribo substitution might affect **5** formation by interfering with this binding. In order to test this possibility, **5** generated in reactions with various substrates was quantitated by HPLC. With the parent DNA bulge substrate 1 (Table 1) the main fluorescent peak at 23.7 min represents product **5** (Fig. 3, arrow a). The minor peaks at 26.5 min and 29 min represent the known spontaneous decomposition products of the drug, **4** and **4a** (Scheme 1), respectively.[2,4,5] With the DNA/RNA hybrid having a ribo a–u substitution on the 5′-side of the target T (duplex 2, Table 3) there is a marked reduction in **5** (arrow c) with a concomitant increase in the degradation products **4** and **4a**. On the other hand, a ribo a–u substitution on the 3′-side (duplex 4, Table 3) has only a small effect on **5** formation (arrow b). The results summarized in Table 3 show that there is a parallel between cleavage efficiency and **5** generation. Furthermore, time courses of **5** formation with the parent DNA duplex and the hybrid duplex are virtually identical (data not shown).

Effect of ribosubstitution on NCS chrom protection against degradation

NCS chrom is a highly labile compound, which degrades rapidly in aqueous solutions at pH > 5. Binding to DNA substrates protects it against degradation; the better the binding, the more efficient the protection.[1] The results obtained on the effect of ribosubstitution on strand cleavage and **5** formation would suggest less efficient binding, hence less protection, of the drug by poorly cleaved DNA/RNA hybrids. It is also possible that the duplex will bind the drug and protect it, but the mode of binding is in a conformation

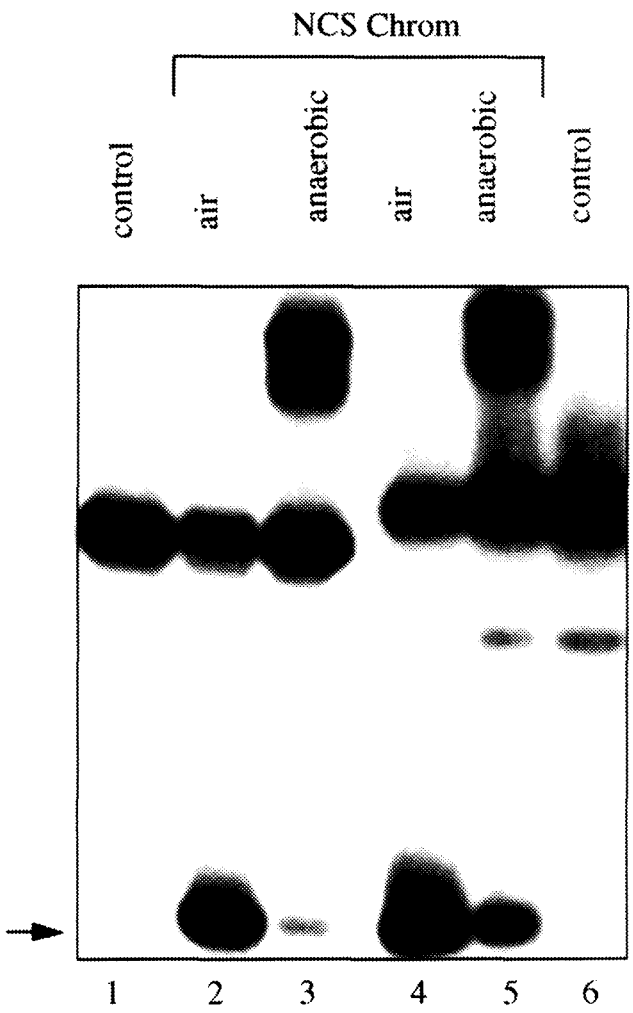


Figure 2. Effect of ribosubstitution in a DNA bulge duplex on the formation of adducts under anaerobic conditions. Standard drug reactions containing DNA bulge duplex or DNA/RNA hybrid bulge duplex were analyzed by HPLC as described in the Experimental section. Lanes 1–3 have DNA duplex 1 and lanes 4–6 have hybrid duplex 2 of Table 2. Arrow indicates the band resulting from cleavage at the T in the bulge.

less conducive to cleavage and **5** formation. In order to distinguish between these two possibilities we determined the extent of protection by the various duplex substrates of NCS chrom against degradation. The assay is based on the generation of drug degradation products as measured by their characteristic fluorescence as a function of time. The duplexes selected for these experiments are those in Table 3, which, on ribosubstitution, showed marked differences in cleavability and product **5** formation. The data in Figure 4 show that the parent DNA duplex 1, which is the best substrate for cleavage and **5** formation, affords the best protection ($k_1 = 0.46 \times 10^{-3}$). On the other hand, hybrid duplex 2, which is only 10–15% as good as duplex 1 in cleavage and generation of **5**, has a k_1 (1.78×10^{-3}), about four times higher than that of duplex 1. Thus, those hybrid duplexes that are poorly cleaved are less efficient in protecting the drug against degradation, implying poor binding of the drug.

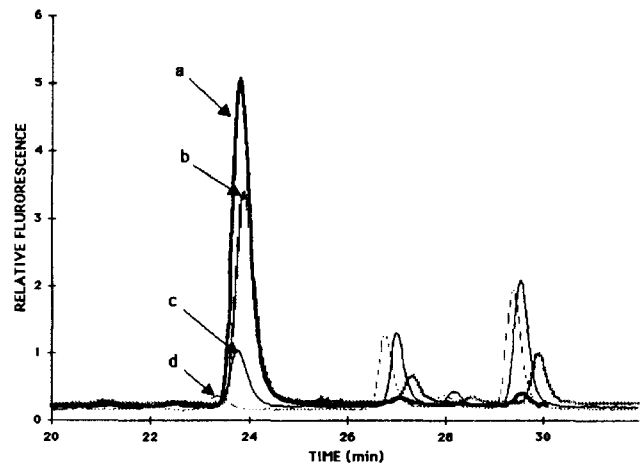


Figure 3. Effect of ribo substitution on the formation of drug product **5**. Standard reactions, containing the indicated duplex substrates (22 μM) and NCS chrom (22 μM), were analyzed by HPLC as described in the Experimental section. Arrow d represents **5** formed by the base-catalyzed degradation of NCS chrom in the absence of DNA or RNA. Not shown is the UV absorbance profile where there is also a distinct peak coincident with the fluorescence peak at 23.7 min.

Discussion

In contrast with DNA (where NCS chrom induced very efficient sequence-specific damage in duplex substrates in the presence of a thiol activator, or site-specific cleavage in DNA bulges in the absence of a thiol activator), in RNAs that presumably contain duplex regions, bulges/loops were cleaved poorly or not at all in either of the mechanistically different kinds of drug reactions.[6,7,9] Further, in experiments in which yeast $tRNA^{Phe}$ was used as substrate we failed to find significant drug-induced cleavage, whether thiol was present[9] or absent (unpublished data). When cleavage was found (primarily at G residues and especially when holo NCS was used) we were not able to eliminate the possibility of contamination of the NCS by nucleases (unpublished data). A recent report from Sugiura's laboratory, however, claims that NCS chrom strongly cleaved yeast $tRNA^{Phe}$ at 5′-G purine steps, although the reaction conditions were not specified and the actual data were not shown.[10] It appears that a much higher ratio of drug to RNA substrate was used in these experiments than in ours; perhaps this accounts for the difference in findings. On the other hand, we found fairly strong sequence-specific damage in the RNA strand of a DNA–RNA hybrid by thiol-activated NCS chrom.[9] There has also been a recent report of single-strand cleavage in high specific activity labeled $tRNA^{His}$ precursor and RNA hairpins by NCS in the absence of thiol, although it is not clear whether isolated chromophore was used.[11] It is rather surprising that these workers found no difference in the cleavage on addition of thiol, since the active radical species is different for the two drug activation mechanisms. Since thiol reacts very rapidly with NCS chrom to form the thiol-activated species to the exclusion of the intramolecularly generated radical species,[3] virtually none of the latter would

Table 3. Effect of ribonucleotide substitution on cleavage and drug product formation

Substrate	Duplex	Strand cleavage (%)	Drug product (%)
1	5′-CCGATGCG 3′-GGCTACGC **T**G	100	100
2	5′-CCGAuGCG 3′-GGCTaCGC **T**G	10	14
3	5′-CCGATGCG 3′-GGCTaCGC **T**G	39	47
4	5′-CCGaTGCG 3′-GGCuACGC **T**G	80	62
5	5′-CCGaTGCG 3′-GGCTACGC **T**G	100	100
6	5′-ccgATgcg 3′-ggcTAcgc **T**G	44	29
7	5′-ccgaugcg 3′-GGCTACGC **T**G	4	5
8	5′-CCGATGCG 3′-ggcuacgc **u**g	2	2

In experiments similar to those in Tables 1 and 2, strand cleavage and drug product were quantitated. Experiments with duplexes 7 and 8 are the same as in Table 1 and Figure 1. Strand cleavage occurs at the bold and underlined residue.

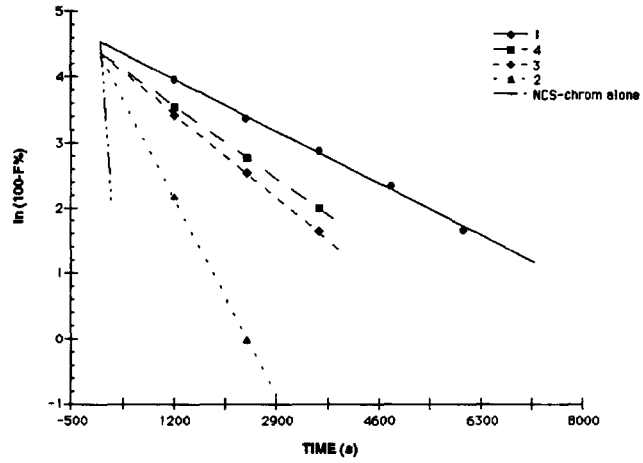


Figure 4. Protection of NCS chrom against degradation. The generation of drug degradation products was monitored by fluorescence spectroscopy as described in the Experimental section. Duplex numbers refer to those in Table 3. The $k_1 \times 10^{-3}$ values are: duplex 1, 0.46; duplex 2, 1.78; duplex 3, 0.76; duplex 4, 0.66. In the absence of any substrate NCS chrom degraded very rapidly ($k_1 = 13.6 \times 10^{-3}$).

be formed in the presence of thiol. Yet, this is the species found to cleave at DNA and TAR RNA bulges.[6]

In the case of TAR RNA the site-specific, base-catalyzed cleavage at the bulge by NCS chrom in the absence of any thiol activator was much weaker than that in its DNA counterpart.[6] Since the DNA bulge created by annealing an 8-mer and a 10-mer (duplex 1, Table 1) has a single site for efficient cleavage by NCS chrom, it provides a simple system suitable for the study of the parameters that govern the structural differences between DNA and RNA by substituting the deoxyriboresidues with their ribo counterparts and following the consequent effects on strand cleavage and generation of the novel drug product. It was found that substitution of a single A–T base pair with ribo A–U on the 5′-side (but not of that on the 3′-side) of the target T at the bulge results in nearly 90% reduction in both strand cleavage and formation of the novel drug product. This clearly shows that NCS chrom can be a valuable tool to probe subtle changes in the microstructure of nucleic acids.

These results coupled with the poor protection by this hybrid of NCS chrom against degradation suggest that a single ribo (A–U) base pair drastically altered the microstructure at the bulge region so as to interfere with drug binding/positioning of the drug causing both inhibition of strand cleavage and generation of the bulge-specific drug product **5.**

The structure of the complex formed between a bulged DNA substrate and compound **4** of Scheme 1, which is an analogue of the proposed DNA cleaving species of NCS chrom, has been determined using 2-D NMR spectroscopy.[12] This study reveals an unusual mode of binding involving tight fitting of the wedge-shaped drug into the triangular prism bulge site and major groove recognition by the carbohydrate unit of the drug. The two drug rings mimic helical bases, which mediate the helical transition between the helices on either side of the bulge. The naphthoate ring stacks mainly on the A residue of the base pair on the 5′-side of the target residue. Thus it appears that the presence of a 2′-hydroxyl group on the sugar of this A residue would sterically clash with the 7″-*O*-methyl group of the naphthoate moiety of the chromophore, accounting for the marked effect of this ribo substitution on cleavage and drug binding. On the other hand, the A–T base pair on the 3′-side of the bulge would be a less important determinant in the bulge-specific drug activity. The findings that ribo substitution does not alter the site-specificity, chemistry of cleavage, or the drug product suggest that the overall binding and mode of interaction of the drug with the DNA/RNA hybrid is the same as with the DNA/DNA duplex. The general decrease in cleavage and binding in the RNA/DNA hybrids (and in the RNA/RNA molecule) not specifically attributed to the above-mentioned steric clash, may be due to the change from a B-conformation to an A-conformation, especially at the junction between hybrid and duplex regions.[13] This is in contrast to the earlier observation that the introduction of mismatched base pairs in a Watson–Crick base-paired DNA duplex causes a switch in the chemistry of damage by NCS chrom in the thiol-dependent reaction.[14] In the latter case the minor groove binding of the drug is via an intercalative

mechanism[1] and results in an induced mutual fit of DNA and drug, largely governed by hydrophobic forces and solvation of the polar surface of the complex.[15]

The present study further extends the usefulness of NCS chrom as a valuable tool for probing the subtle structural perturbations in nucleic acids. In addition, site-specific cleavage at the bulge is of interest because of the involvement of bulged structures in a variety of biological processes.[16–19] Further, the availability of a 3-D structure of the drug·DNA bulge complex[12] should provide insight into the design of agents with greater RNA bulge specificity.

Experimental

Nucleic acid substrates

Oligodeoxyribonucleotides were synthesized by phosphoramidite chemistry using an Applied Biosystems DNA synthesizer. Oligoribonucleotides were similarly synthesized and purified on a 15% denaturing polyacrylamide gel. Radioactive materials and enzymes were purchased from New England Nuclear and New England Biolabs, respectively. Oligomers were 5′-end labeled with ^{32}P by standard procedures[20] and purified by electrophoresis on a 15% sequencing gel. Neocarzinostatin powder (holo NCS) was obtained from Kayaku Antibiotics (Tokyo).

Drug reaction

NCS-chrom was extracted from the holoantibiotic by cold methanol containing 0.5 M acetic acid by a procedure similar to that described by Myers.[21] The chromophore was stored at −70 °C protected against light. The two strands of the oligomers were annealed by heating in the reaction buffer (Tris–HCl pH, 8.6) at 70 °C for 2 min and cooling slowly to room temperature. In some cases the heating step was omitted; this did not affect the strand cleavage specificity or intensity. The mixture was cooled in ice for 15 min prior to the addition of NCS chrom. The final reaction (60 μL) contained 50 mM Tris–HCl pH, 8.6, ^{32}P-labeled 10-mer, annealed with an excess (two- to 2.5-fold) of the 8-mer, and NCS chrom at concentrations given in the figure legends. The reaction was allowed to proceed in the dark for 1 h in ice. Maximum final methanol concentration was 10%.

Anaerobic drug reactions (150 μL) were performed in a reaction vessel equipped with a side arm, as previously described.[22] The procedure was modified so as to permit the removal of oxygen from the reaction vessel under conditions where the drug will be stabilized by binding to the substrate, but the damage is minimal or not at all, until it is initiated by raising the pH. The oligomers were placed in the main chamber in Na acetate, pH 5.0 (15 mM) and cooled in ice. After the addition of chromophore the mixture was frozen and evacuated. Evacuation was repeated four times with intermittent thawing and freezing. The pH was then raised to 8.6 by mixing the components of the main chamber with the Tris–HCl buffer, pH 9.0, present in the side arm.

Product analyses

In order to determine strand cleavage, portions of the reaction mixture were dried, and the sample pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes and analyzed on a 15% sequencing gel. The gel band intensities were quantitated on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

The novel drug product formed in the reaction was separated by reverse-phase HPLC using an analytical C18 column (Waters) and a linear gradient (1 mL/min) of methanol/H_2O containing 10 mM ammonium acetate, pH 4.8. Prior to injection of the sample into the column, the reaction mixture was treated with nuclease S1 to convert the oligonucleotides to monomers.[20] Absorbance (254 nm) and fluorescence (excitation at 400 nm, emission at 550 nm) of the column eluate were constantly monitored.

Protection of NCS chrom against degradation

Drug binding to substrate was assessed by the ability of an oligomer duplex to protect the drug from degradation by following the time course of the increase in fluorescence (excitation at 400 nm; emission at 550 nm) attributable to the drug degradation products. The complete mixture (500 μL) contained 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 10 μM bulge strand (10-mer), 20 mM 8-mer and 5 μM NCS chrom. NCS chrom was added last to the cuvette containing the rest of the components precooled to 0 °C, and fluorescence measurements were made.

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