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Binding and Cleavage Characteristics of the Complexes Formed Between the Neocarzinostatin Chromophore and Single Site Containing Oligonucleotides

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Abstract—It is shown by fluorescence spectroscopy that the post-activated form of neocarzinostatin chromophore (NCSi-glu) can form stable complexes with single-site oligonucleotides (SSOs) featuring sequences known to be involved in double stranded (AGC·GCT, AGT·ACT, AGA·TCT, ACA·TGT) or single stranded (AGG·CCT) cleavage (attacked residues in bold). Furthermore, the same SSOs form cleavage productive complexes with native neocarzinostatin chromophore (NCS chrom) over a similar concentration range. The productive complexes yield damage similar to that observed if the same sequence is part of a longer DNA piece. Previously identified double stranded site sequences ATT·AAT and TAT·ATA are shown to contain overlapping attack sites. Binding order preference derived from fluorescence quenching experiments for NCSi-glu is consistent with constants derived by quantitative cleavage affinity binding experiments with NCS chrom. This confirms the similarity in interactions between the NCSi-glu and NCS chrom and justifies the use of NCSi-glu as a stable analog of NCS chrom.

Introduction

The neocarzinostatin chromophore (NCS chrom) (**1**, Fig. 1), prototype of the growing family of potent enediyne antitumor antibiotics, has been studied for almost 30 years.[1,2] As is true for all enediynes, NCS chrom's mode of action is believed to be through hydrogen atom abstractions from deoxyriboses in the minor groove of DNA resulting in both direct and indirect (alkali-dependent) strand breaks. NCS chrom has been shown to induce single (ss) and double stranded (ds) DNA lesions *in vivo* (5:1) and *in vitro* (6:1 direct, 2:1 including indirect), after activation by the thiol naturally occurring in cells, glutathione.[2] NCS chrom's mechanism of activation by glutathione is thought to involve a nucleophilic attack of the thiolate group at C-12, which results in the formation of a cumulene intermediate (**2**) (Fig. 1).[2,3] The cumulene spontaneously undergoes a Bergman-like rearrangement to a reactive biradical form of the drug (**3**), which is responsible for the hydrogen abstractions. The final product of the drug transformation is the post-activated aromatic form of the thiol adduct (NCSi-thiol, **4**).[2]

In vitro and *in vivo* studies have demonstrated that NCS chrom will generate DNA lesions in an oxygen and thiol dependent fashion with ss breaks occuring primarily at T and A residues (T > A >> C > G) and ds breaks in a sequence specific manner.[2,4] Double stranded lesions are thought to be responsible for neocarzinostatin's cytotoxicity, since they are difficult to repair and can be mutagenic in nature.[2,4a] The majority of the ds lesions are found at the trinucleotide sequences AGT·ACT and AGC·GCT (with the attacked residues in bold) and are both direct and base-induced.[5,6] Whereas the strand breaks at the **T** residues involve mainly H-5' abstraction, the lesions at the **T** of AGT and the **C** of AGC involve H-4' and H-1' abstractions, respectively, which produce strand breaks and/or abasic sites.[4–6]

Studies have shown that the ds breaks are due to specific interaction of one molecule of activated chromophore per site.[6b,c] A structural model for the AGC·GCT ds cleavage site has been proposed based on molecular mechanics and molecular dynamics calculations, which was able to explain the binding and cutting specificity at that site.[7] Subsequent experiments with specifically deuterium-labeled self-complementary oligonucleotides confirmed the model by establishing which carbon radical of the biradical form of the drug was responsible for each particular hydrogen atom abstraction at that site.[8]

Characterization of drug–DNA complexes for the ds breakage-sites could provide insight not only into the difference in reactivity between these sites but into the specific recognition code of the drug, as well. Unfortunately, physical characterization of the complex of NCS chrom with a longer piece of DNA is complicated by the promiscuity of the drug, which results in many binding sites leading mostly to ss cutting.[2] Furthermore, the well documented lability of the native NCS chrom in aqueous solutions complicates matters even more.[9] Use of a minimal piece of DNA, where a trinucleotide binding site is flanked by G:C pairs that do not get damaged by NCS chrom (single-site-oligonucleotides, SSOs), circumvents this problem. Positioning of the binding/cutting site in the middle of the SSO further ensures its interaction with the drug,

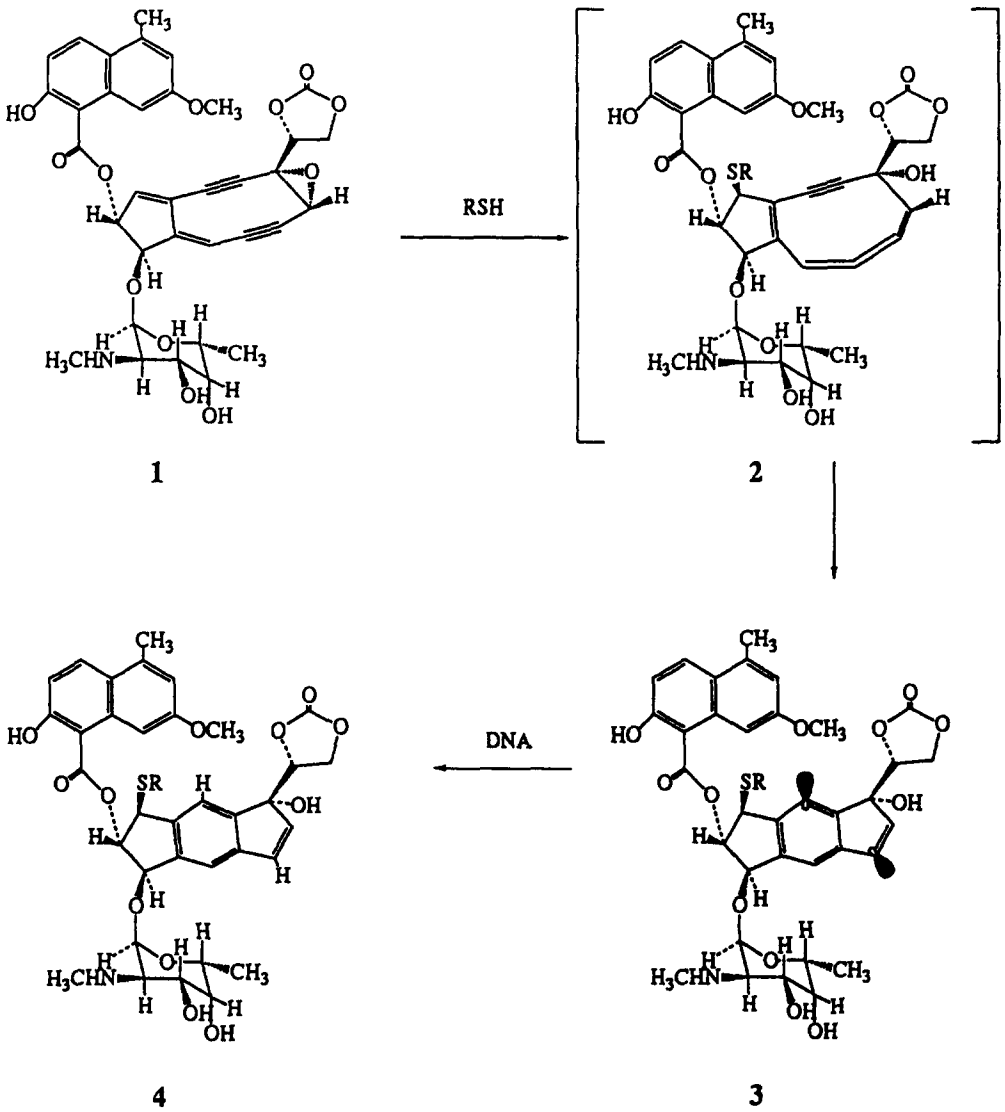

Figure 1. Reaction scheme for the activation of NCS chrom by glutathione.[3]

since fraying effects would destabilize non-specific interactions with the sequences at either end. Replacement of the native by the glutathione post-activated form of the drug (NCSi-glu), known to bind to ds DNA,[9b] overcomes the stability problem. This allows the use of techniques that require significant lifetime of the resulting complex, e.g. ^{1}H NMR, crystallography.

Use of NCSi-glu (**4**) as an analog is justified by its structural similarity to the biradical form (**3**) of the drug, proposed to be responsible for the hydrogen atom abstractions. Additionally, both NCS chrom (**1**) and NCSi-glu (**4**) feature the same groups important for DNA binding, namely the intercalating naphthoate and the *N*-methyl fucosamine moieties. The critical role of these moieties in determining the site of binding has been demonstrated in the ^{1}H NMR solution structure of the complex between NCSi-glu and the AGC·GCT site.[10] Finally, the glutathione post-activated form is the most appropriate post-activated form for analyses of drug–DNA complexes, since the nature of the thiol affects sequence specific DNA damage,[6a] and glutathione is the activating thiol *in vivo*.

We report here the cleavage patterns and binding characteristics for a series of SSOs containing the most common ds-lesion sites for NCS chrom and NCSi-glu. Although it is reasonable that binding affinities and cleavage specificities might go hand-in-hand, it also seems possible that detailed geometric configuration at the site may be critical in differentially influencing the two types of interaction parameters. These studies show that affinity measured by the fluorescence properties of the drug–SSO complex is consistent with that determined by the quantitative affinity binding assay (QAB).

Results and Discussion

NCS chrom shows a remarkable selectivity despite its small size (MW < 1000, length equivalent of 3–4 bp), which limits the possible interactions with linear molecules like ds DNA. Owing to this relatively low selectivity several attack sites can be found even for small oligonucleotides of random sequences, mostly leading to ss lesions.[2] In order to avoid multiple sites

one needs to resort to oligonucleotides of small size (7-mers). A series of complexes have been characterized between NCS chrom or its analog NCSi-glu and heptanucleotide SSOs of the general formula:

5'dGGXYZGC3'
3'dCCPQRCG5'

for the sites found to give ds damage with NCS, where XYZ base pairs with the RQP sequence (Table 1). The ds sites selected included not only the most prominent ones (AGC·GCT and AGT·ACT),[2] but also minor ones identified recently (ACA·TGT, AAT·ATT, AGA·TCT, ACC·GGT, TAT·ATA).[6b] Finally, the SSOs containing GCG·CGC and AGG·TCC sites were selected as examples of a non-binding sequence and a ss break site, respectively.

Cleavage reactions

Standard drug reactions on the SSO sequences showed the cleavage patterns depicted by bold characters in Table 1. The AGC·GCT and AGT·ACT sites exhibited the expected patterns observed in the past for these sequences.[4-6] When the two strands of these SSOs (Table 1; entries 1, 2) were 5'-P^{32}-end-labeled in a side-by-side experiment, cleavage occurred on both strands at ratios almost equal to 1:1 (C/T = 0.91, T/T = 0.81) indicating that essentially only ds cleavage occurred. This equilateral cleavage persisted over three orders of magnitude of NCS chrom concentration change. This result is in contrast to previous observations for the AGC·GCT sequence, where the attack at the T position exceeded the attack at the C position by a factor of about 4.[5] Treatment with base was necessary for the complete expression of the strand cleavage at C in AGC·GCT, indicating the formation of an abasic site as one might expect for this site.[5] The same extent of cleavage was observed for AGC·GCT and AGC·ICT (Table 1; entries 2, 3), indicating that replacement of the guanine on the (–) strand with an inosine, in contrast to earlier findings,[5a] does not enhance the formation of an abasic site at the AGC site. These differences with previous observations may be a result of the small length of the SSOs or a result of the fact that the oligonucleotide studied is not self-complementary. In the past quantitations of the damage at the T versus the damage at the C were carried out on self-complementary oligonucleotides, where simultaneous observation of the cuts at the two sites is possible.[5,8] It is conceivable that in a self-complementary oligonucleotide, where the two ds-binding/cutting sites are close together, interference of the two molecules of drug with one another may influence the ds/ss cutting ratio for these sites. In a control experiment with the self-complementary oligonucleotide 5'GGAGCGCGCTCC3' the predominant damage at the T over the one at C was confirmed (T/C ≈ 4). On the other hand, when the non-complementary [AGC/AGT] double-site oligonucleotide (Table 1, entry 10) was investigated, the ratio of damage on the two strands (after basic treatment) was found to be about 1:1 for the AGT site and about 2:1 for the AGC site (T/C). These ratios were also found to depend on the drug/DNA ratio. For the AGT site the percentage of ds cleavage was found to decrease with decreasing drug concentration deviating (percentage decreased from 100 to 60% upon decrease of the drug concentration by two orders of magnitude), while for the AGC site the percentage of ds cleavage was found to increase with decreasing drug concentration (percentage increased from 50 to 75% upon the decrease of the drug concentration by two orders of magnitude). Thus, the extent of ds lesions compared with the lesions at a particular site depends not only on sequence but on the drug concentration and other characteristics of the oligonucleotide.

Table 1. Dissociation constants from quantitative affinity binding measurements

Entry	Oligonucleotides[1]	K_d[2]	
1	5'GGAGTGC 3'CCTCACG	1.4	
2	5'GGAGCGC 3'CCTCGCG	3.9	
3	5'GGAGCGC 3'CCTCICG	3.7	
4	5'GGACAGC 3'CCTGTCG	2.2	
5	5'GGAGAGC 3'CCTCTCG	3.8	
6	5'GGAATGC 3'CCTTACG	4.8	
7	5'GGTATGC 3'CCATACG	5.1 3.8	
8	5'GGGCGGC 3'CCCGCCG	nd[3]	
9	5'GGAGGGC 3'CCTCCCG	4.7	
10	5'GGAGCGCGGAGTGC 3'CCTCGCGCCTCACG	2.8	0.7[4]

[1]The residues attacked are in bold lettering.
[2]K_ds correspond to damage at the strand aligned with the number; in the case of multiple sites, only the tighter binding is reported.
[3]No cutting detected.
[4]The dissociation constants correspond to the sites in the 5'-3' direction.

The ACA·TGT and AGA·TCT sites (Table 1; entries 4,5) exhibited the standard ds cleavage where the attacked residues are two bp apart in the 3' direction, while sequences that contained multiple As and Ts, (Table 1; entries 6,7) proved to be multiple targets for both ss and ds cleavage. This was indicated by the additional cleavage in the middle nucleotides of AAT·ATT and all three nucleotides of ATA·TAT. These cleavage patterns cannot be explained by attack from a single molecule of NCS chrom, but rather multiple events need to be invoked. Alternatively, the

other binding events take place on a different trinucleotide region, frame-shifted from the intended one.

Labeling of both strands of the AGG·CCT site in a side-by-side experiment demonstrated the ss cleavage nature of the site, with cutting observed on the TCC strand only. The yield of strand breakage at the T is not enhanced after treatment with piperidine, consistent with H-5' abstraction. Finally the GCG·CGC SSO showed no specific cleavage even at high drug/DNA ratios. At the highest drug concentrations low level non-specific cutting could be observed throughout the sequence.

Quantitative affinity binding

Since cleavage of DNA must, by definition, be preceded by association with the drug, it is possible to obtain a binding constant for an individual site by applying the principles of quantitative affinity cleavage[11] to the NCS chrom cutting reaction. This assumes the simplified equilibrium in equation (1).

$$\text{SSO} + \text{NCS chrom} \rightleftharpoons \text{BC} \rightarrow \text{cleavage products}, \quad (1)$$

where BC is the productive binding complex. By the use of only a small amount of 5'-^{32}P-end-labeled strand (< 5% of the lowest drug concentration) annealed to a slight excess of the other strand and variable concentrations of NCS chrom, we were able to measure the dissociation constants (Table 1). Figure 2 shows a representative set of data for the ACA·TGT site reaction. The dissociation constants obtained describe the binding of the species responsible for the specificity of DNA cutting. This species has been proposed to be the cumulene intermediate of the NCS chrom activation by thiol.[12] It has also been shown that the preformed cumulene shows identical sequence specificity as the *in situ* generated intermediate for a 35 bp long restriction fragment.[12b] Given the similarity of the DNA recognizing groups, hydroxynaphthoate and *N*-methyl fucosamine, it seems likely that both NCS chrom and the cumulene will recognize the same sequences. For all practical purposes the dissociation constant determined by the QAB method can, therefore, be attributed to NCS chrom. The values are all in the low μM region, which roughly agrees with the statistical binding constant obtained in the past for NCS chrom ($K_d \approx 5$ μM) with calf thymus DNA (CT DNA).[13]

The QAB method is based on the assumption that the cleaving efficiency of the agent used, in this case NCS chrom, is proportional to the fraction of the agent bound on the labeled DNA. In the case of NCS chrom that has complex decomposition patterns competing with the DNA cleaving reaction, the cleaving efficiency might deviate from this behavior. These values provide, therefore, a higher limit for the actual dissociation constants, in the same way the Michaelis–Menten constants provide a higher limit for the substrate enzyme binding constant.[14] Each lesion on the oligonucleotides studied can yield information about the species causing it. Lesions caused by the same species/event should yield similar dissociation constants. If the concentration dependence of two independent lesions observed are similar, then the two events cannot be distinguished by this method.

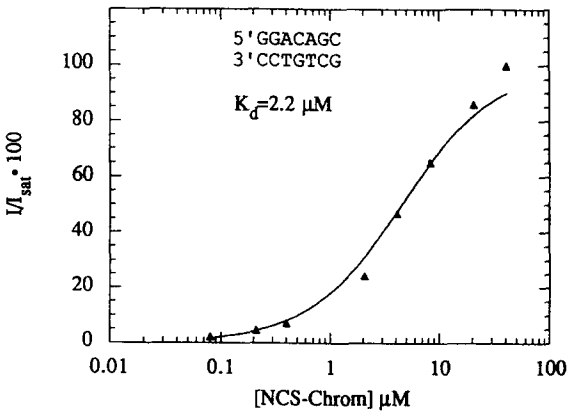

Figure 2. Quantitative affinity cleavage experiments involving the ACA·TGT oligonucleotide. The data points represent the signal intensities (I) for the cleavage band at each NCS chrom concentration, normalized versus $I_{sat} = 100$.[11] Equation 2 was used to fit the data and the solid line represents the best-fit binding titration isotherm.

The average dissociation constants for ds lesions (Table 1) were observed to follow the trend:

$$\text{AGT·ACT} < \text{ACA·TGT} < \text{AGA·TCT} \approx \text{AGC·GCT}.$$

This trend may explain why AGT ds lesions predominate in ds DNA. As mentioned above, multiple attacked residues were observed for the lesions at the **AAT·ATT** and **TAT·ATA** SSOs. Moreover, different damage sites showed a different concentration dependence indicating different dissociation constants for the species causing them. More specifically, for **AAT·ATT** the attack at the two Ts at the ends of the sequence on the 3' and 5' directions had similar concentration dependence, corresponding to a dissociation constant of 4.8 μM. On the other hand, the attack at the second residue of the triplets appeared only on higher concentrations and did not saturate at the range of concentrations studied. For the **TAT·ATA** sequence, the attacks at the second and third residues of each trinucleotide had similar concentration dependence ($K_d \approx 5$ μM), while the attack at the last residues appeared at higher concentrations only and did not saturate at the range of concentrations studied. For both sites AAT and TAT a lower limit for a dissociation of ~11 μM can be calculated from the concentration dependence of the secondary cleavage. Given the biradical mechanism of NCS action, only a maximum of two breaks resulting on bases two bp apart can be justified as a double cut for one molecule of drug. This leads us to conclude that the AAT and TAT sites are not straightforward ds cleavage sequences but rather the result of multiple attacks by the drug. It is intriguing

that these sequences feature multiple AT pairs, which are also known to be sites of ss lesions for NCS. A plausible explanation for the multiple attack sites is that there are several possible drug–SSO (1:1) complexes that coexist, giving rise to the different lesions. On the other hand, the possibility exists that more than one drug simultaneously bind the same oligonucleotide, although it is not obvious how the two drug molecules might be accommodated in the limited space available. This would imply a different mode of binding associated with ss attack and/or the concerted attack of two NCS chrom molecules at the same site. Indeed, the mode of binding proposed for the AGC site,[7] which was confirmed by the ^{1}H NMR data,[10] cannot explain the presence of multiple binding sites in such a small space.

For the ss break site AGG·CCT, a binding constant of 4.7 μM was obtained which is comparable to the ds cleavage binding. This, in association with the higher statistical possibility of ss sites in a random sequence could explain their high incidence. Finally, since no cutting was observed for the GCG·CGC sequence no dissociation constant could be obtained by this method.

Fluorescence quenching

Binding of both the NCS chrom and NCSi-glu results in quenching of the fluorescence at 440 nm with long pieces of DNA.[9b] The same behavior was observed with the SSOs. Association of the SSOs with the native NCS chrom was followed by fluorescence spectroscopy at low temperature (~0 °C) and low pH (pH ≈ 5).[13b] Although some fluorescence quenching could be detected in the presence of the AGC·GCT or AGC·ICT at 440 nm, parallel decomposition of the drug could be observed as indicated by the increase of fluorescence at 490 nm (λ_{exc} = 360 nm).[9] This has been shown to be associated with the production of the base-catalyzed decomposition products.[15] These experiments confirmed the lability of NCS chrom even under these mild conditions. When the non-binding control GCG was used, faster decomposition and no significant fluorescence quenching at 440 nm was observed with the non-binding control GCG SSO. This behavior is reminiscent of the NCS chrom protection from spontaneous decomposition by DNA binding.[9b]

Characterization, on the other hand, of stable complexes between the SSOs and the NCSi-glu was possible under physiological conditions (pH 7.5) via fluorescence quenching. Figures 3 and 4 show representative results for the AGC·GCT and AGT·ACT sequences. As in the case of CT DNA, binding of NCSi-glu to the SSOs was accompanied by quenching of the fluorescence at 440 nm.[9b] Standard Scatchard analysis[16] (Fig. 4) of the binding isothermic curves (Fig. 3) for the SSOs gave values in the μM range (Table 2) and showed a single type of binding.

In a similar fluorescence quenching experiment with CT DNA, (a heterogeneous mixture of long DNA pieces approximately 300 bp long) (Fig. 5) NCSi-glu exhibited two kinds of binding, a tight region with $K_d \approx 0.4$ μM (type I) and a weak binding region with a K_d of ~20 μM (type II). We attribute the type I binding to site-specific interactions, while the type II binding can be attributed to either non-specific intercalation or the binding of self-stacked molecules of the cationic drug to the polyanionic DNA.[17] Similar biphasic behavior has been observed for the intercalator proflavine in the classic study of Peacocke and Skerett.[17b] Direct comparison of

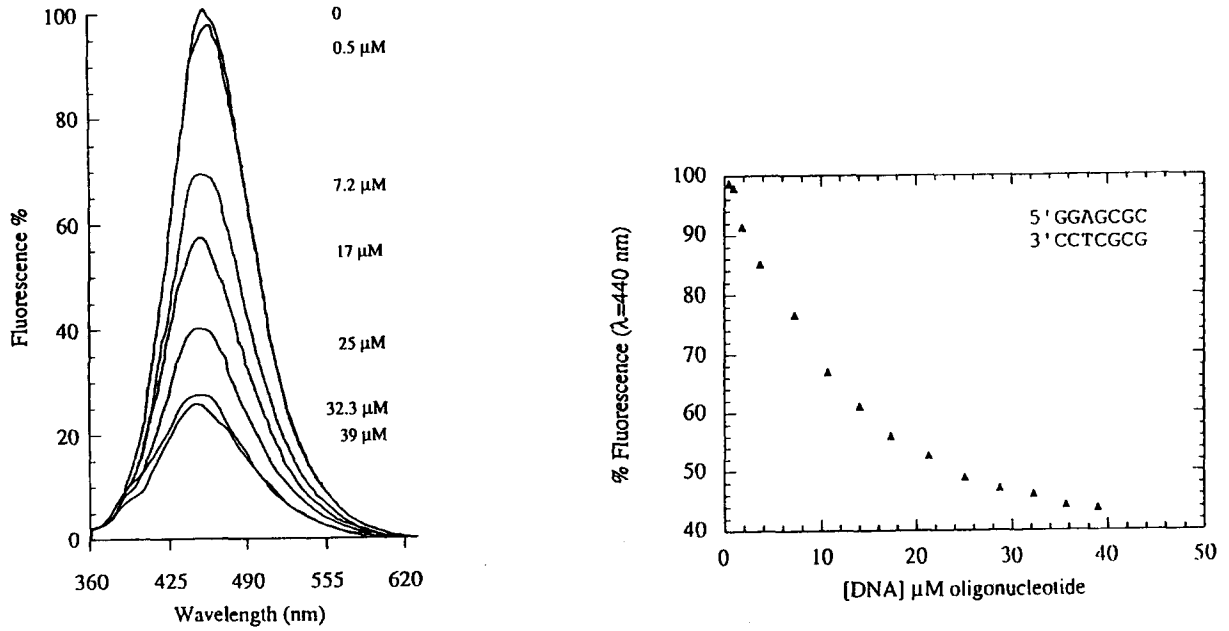

Figure 3. Binding of NCSi-glu to the AGC·GCT containing SSO. (Left) Fluorescence spectra of NCSi-glu in the presence of increasing amounts of annealed AGC·GCT in 10 mM Tris–HCl, pH 7.5 and 10% MeOH. Excitation wavelength is 360 nm. Concentration expressed in moles of oligonucleotide. Final NCSi-glu concentration was ~0.5 μM. (Right) Normalized fluorescence intensity (440 nm; λ_{Exc} = 360 nm) for NCSi-glu in the presence of increasing amounts of annealed AGC·GCT versus oligonucleotide concentration.

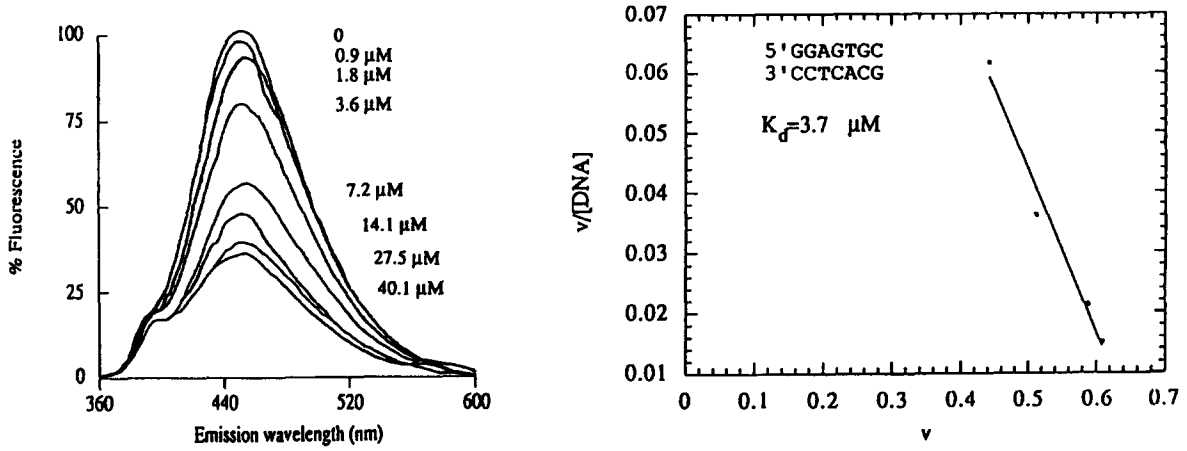

Figure 4. Binding of NCSi-glu to the AGT·ACT containing SSO. (Left) Fluorescence spectra of NCSi-glu in the presence of increasing amounts of annealed AGT·ACT in 10 mM Tris–HCl, pH 7.5 and 10% MeOH. Excitation wavelength is 360 nm. Concentration expressed in moles of oligo. Final drug concentration was ~0.5 μM. (Right) Scatchard plot (v/[DNA] versus v) of NCSi-glu binding to the AGT·ACT SSO, for the data presented in Figure 4a. v is the fraction of DNA binding sites occupied by molecules of the drug and [DNA] is the concentration of duplex oligomer in moles. The data points based on the corrected (for dilution) fluorescence were fitted to equation (3). The solid line is the least square linear fit ($y = 0.1785 - 0.26906x$; $R = 0.98$).

Table 2. Dissociation constants for the NCSi-glu from fluorescence quenching measurements

Entry	Oligonucleotides[1]	K_d
1	5'GGAGTGC 3'CCTCACG	3.7 μM
2	5'GGAGCGC 3'CCTCGCG	15.8 μM
3	5'GGAGCGC 3'CCTCICG	22.2 μM
4	5'GGACAGC 3'CCTGTCG	10.2 μM
5	5'GGAGAGC 3'CCTCTCG	14.5 μM
6	5'GGAATGC 3'CCTTACG	2.2 μM
7	5'GGTATGC 3'CCATACG	3.2 μM
8	5'GGGCGGC 3'CCCGCCG	≈60 μM[2]
9	5'GGAGGGC 3'CCTCCCG	11.1 μM

[1]The residues attacked during drug reactions are in bold lettering.
[2]The fluorescence curve did not show saturation behavior even at the highest concentration of oligonucleotide.

the dissociation constants in Table 1 to the value obtained for the statistical interaction of NCSi-glu with the heterogeneous mixture of sites on long pieces of CT DNA, shows the SSO values to be larger than the average values of the type I interactions. The difference can be attributed to the short length of these oligonucleotides and therefore the lower stability of complexes, when compared to the complexes formed with the ~300 bp length of CT DNA. It is interesting to note that unlike the other oligonucleotides, the non-target GCG·CGC SSO shows a remarkably higher dissociation constant (60 μM). This value is in agreement with the cleavage reaction, where no discernible localized cutting could be observed even at high drug/DNA ratios. The total of the binding sites used in this study provides, therefore, a model for both types of interactions observed in physiologically relevant DNA. This different reactivity for the GC rich sequence may also be connected with different DNA microstructures, recognized by the drug.

For all the ds-sites the dissociation constants obtained by the fluorescence quenching method follows the general order:

$$\text{AGT·ACT} < \text{ACA·TGT} < \text{AGA·TCT} \approx \text{AGC·GCT} < \text{AGC·ICT},$$

which is quite similar to the order obtained for the QAB method. Both methods seem to indicate that the binding is tighter at AGT than at AGC sites. In accordance with this cleavage experiments on an oligonucleotide containing both sites (Table 1, entry 10) showed that, when in direct competition with the AGC site, AGT is cleaved more efficiently (higher % of damage). It is intriguing that the replacement of the amino group of guanine with a hydrogen (Table 2, entry 3 versus 2) makes the complex of NCSi-glu with the AGC sequence less tight. This again is opposite to what might be intuitively expected for the substitution which gives a four-fold enhancement of damage at the C residue, as was observed in the past[5a] but not in this study.

Sites AAT·ATT and TAT·ATA showed like dissociation constants of 2.2 and 3.2 μM, respectively, which are lower than the average ds-site dissociation constants. The tight binding may be the result of multiple binding sites with similar constants, which are not mutually exclusive. If this is true, these lower values

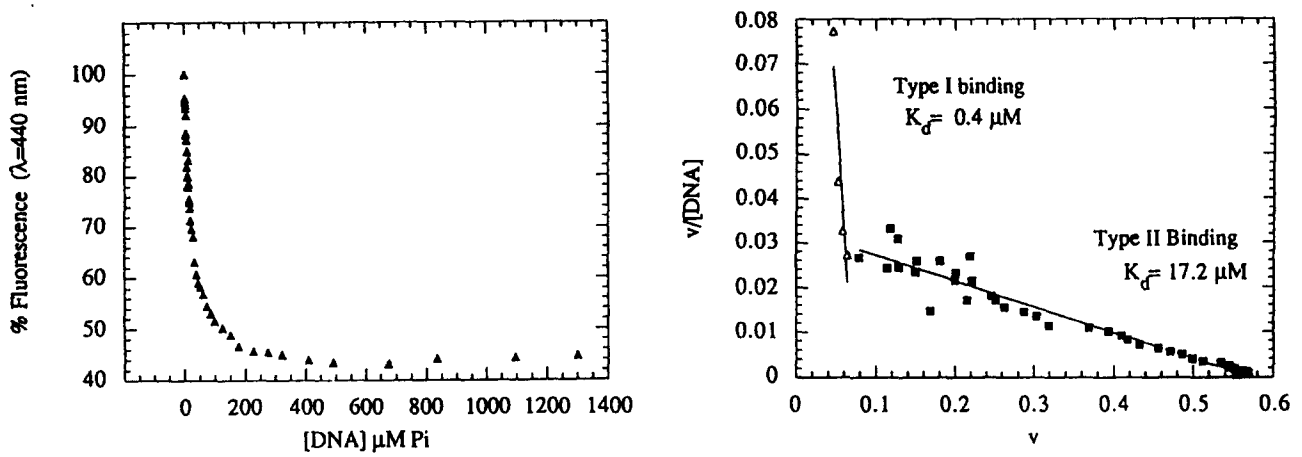

Figure 5. Binding of NCSi-glu to CT DNA. (Left) Normalized fluorescence intensity (440 nm; λ_{Exc} = 360 nm) for NCSi-glu in the presence of increasing amounts of CT DNA versus the DNA concentration (moles of phosphate). Final drug concentration was ~1 μM. (Right) Scatchard plot of NCSi-glu binding to CT DNA, for the data presented in Figure 5a. *v*/[DNA] versus *v*. *v* is the fraction of DNA binding sites occupied by molecules of the drug and [DNA] is the concentration of CT DNA in moles of phosphate. The data points were separated in two regions and the points in each region were fitted according to equation (3). The solid lines are the least square fits of equation (3) for each region.

obtained by the fluorescence quenching method are a consequence of the fact that this method does not depend on the number of bound residues per site as opposed to the QAB method where each residue can give a maximum of two damaged residues. It is interesting to note, however, that these two SSOs are the only ones to exhibit a shift of the fluorescence maximum from 440 to 420 nm at higher concentrations of oligonucleotide (data not shown). This same shift is observed for the interaction between NCSi-glu and CT DNA. A shift of the fluorescence maximum for NCSi-glu was not observed, however, upon binding to any of the other oligonucleotides, indicating that this spectral signature is characteristic of the interaction with this type of sequence.

Finally, fluorescence quenching for the only ss cutting sequence studied was observed in the same manner as for the ds-site SSOs and a dissociation constant of a slightly higher magnitude (11.1 μM) was obtained. The quenching of fluorescence observed argues in favor of a mode of binding similar to the ds-site binding, involving intercalation of the naphthoate moiety. However, it is conceivable that alternative binding modes could also accommodate naphthoate group intercalation and, therefore, similar fluorescence quenching upon binding.[7a]

Comparison of the two methods

Comparison of the dissociation constants for the two different methods for measuring binding shows that they follow the same trends as one might expect for species with similar recognition properties. Differences exist, and it may be that the limitations of the assumptions used to analyze the cutting data are responsible for this discrepancy. It may also be possible that this difference reflects the control of the cleavage reaction by factors other than the thermodynamic stability of the drug/DNA complex. The most probable explanation for the observed differences between the absolute values of the K_ds is the structural difference between the two species studied, the NCS cumulene and the NCSi-glu.

Recent solution of the 3-D structure for the complex between NCSi-glu and the AGC•GCT containing SSO with ^{1}H NMR methods[10] illustrates how the SSO–NCSi-glu complexes can be used to elucidate the 3-D structure of a drug complexed to a specific attack site. This structure proves the formation of a single complex between AGC and NCSi-glu (> 95%), with a structure confirming the model proposed earlier by Galat and Goldberg.[7a] NCSi-glu interacts with the three nucleotides AGC on one strand and four (GTCG) on the other. As expected,[2] minor groove binding and intercalation at the AT/GC step is observed. Specific contacts are observed between the *N*-methylamino sugar group and the adenine H-2, as well as between H-8" on the hydroxynaphthoate group, H-2 and H-11 on the tetrahydroindacene and pro-*S* H-14 protons with the amino groups of the G residues in the recognition sequence. This structure confirms the hydroxynaphthoate and the aminoglycoside groups to be important in determining the specificity of the drug for this attack site. As in the earlier model, the positions and relative orientations of NCSi-glu's C2 and C6 hydrogens in the complex are such as to rationalize the H-1 and H-5 abstractions from the **C** and **T** bases, respectively. Structural elucidation of other complexes is under way.

Conclusion

We have shown that the glutathione post-activated form of NCS-Chrom can form stable complexes with SSOs featuring sequences known to be involved in ds (AGC, AGT, AGA, ACA), or ss (AGG) cleavage. Furthermore, the same SSOs form productive complexes with the native NCS chrom over a similar concentration range. The productive complexes yield damage similar to that observed if the same sequence is part of a longer DNA

piece. Sequences ATT and TAT are shown to be multiple 'hit' sites, where more than one reaction overlap. In all cases binding order preference measured by fluorescent quenching of NCSi-glu corresponds with that determined by cleavage affinity measurement.

The stable complexes formed by NCSi-glu and the SSOs lend themselves to structural elucidation. The structure of an SSO–NCSi-glu (AGC·GCT–NCSi-glu) complex has been recently elucidated by the use of ^{1}H NMR spectroscopy.[10] The structure found not only gives insight as to what groups are important for the binding interactions, but can also explain the hydrogen abstractions observed for the respective productive binding complex of the transient biradical.

Experimental

Reagents

NCS chrom was isolated from holo-NCS (Kayaku Antibiotics) as described[5] and stored as a MeOH solution at –70 °C. NCSi-glu was isolated and purified from a reaction mixture of NCS chrom with glutathione by HPLC, as previously described.[8b]

Oligonucleotides were synthesized on a 381A Applied Biosystems automatic synthesizer, deprotected according to standard procedures with concentrated ammonium hydroxide and then *n*-BuOH precipitated (9:1, v:v) as the ammonium salts, checked for purity and used without further purification. The oligonucleotides were quantified by their A_{260}, using the additive extinction coefficients[18] and their purity confirmed by denaturing gel chromatography (UV shadow).[19] Phosphoramidites, other reagents and solvents were purchased from Glen Research. In order to anneal oligonucleotides for the drug reactions and the fluorescence measurements, equimolar amounts of the two strands (~250 μM strand) were mixed in the appropriate buffer, the solution was heated at 90 °C for 5 min and then allowed to cool to 4 °C slowly.

Methods and data analysis

5'-P^{32}-End-labeling was carried out according to standard procedures.[19] The labeled oligonucleotides were isolated on a 20% denaturing gel; the bands were excised and were isolated by the crush and soak procedure.[19] They were subsequently desalted by the use of a G-25 Sephadex column (Pharmacia). Denaturing gels of the appropriate percentage were made using the SEQUAGEL system (National Diagnostics). CT DNA was bought from Sigma and was sonicated and phenol extracted before extensive dialysis versus the buffer of choice.

NCS chrom cleavage reactions were carried out according to Kappen *et al.*[5] All the components of the reaction were mixed together and incubated on ice for 30 min before the addition of the drug as an acidic methanolic solution. Control experiments contained an equal volume of methanol. The reactions were carried out in Tris–HCl 20 mM, 1 mM EDTA; pH 8.0, 10% MeOH.

In side-by-side experiments equimolar amounts of 5'-^{32}P-end-labeled oligonucleotides of the same specific activity, each labeled on different strands were reacted with the same amount of NCS chrom. The reaction products were analyzed on the same denaturing gel and analyzed with the use of a Phosphorimager.

Quantitative affinity binding measurements were carried out in the method of Singleton and Dervan.[11] The glutathione reaction was used as the cleavage method and the products of the reaction were analyzed on 20% denaturing gels, after treatment with piperidine. The basic treatment (30 min, 90 °C) is necessary to express the damage caused by the drug to its full extent by inducing strand fragmentation where sugar damage exists and by fully denaturing the oligonucleotides. The appropriate bands were analyzed with the use of a Phosphorimager screen (Molecular Dynamics) and a Molecular Dynamics PhosphorImager SP. The integrations of the bands were performed as volume integrations using the ImageQuant v 3.4 software. Data analysis was carried out following the arguments of Singleton and Dervan.[11] The relative cleavage at the appropriate sites was expressed as a percentage of the relative intensity of the band at the concentration yielding saturation and then plotted versus the logarithm of the drug concentrations. These semi logarithmic plots were then fitted to equation (2):

$$I_{site} = I_{sat} \cdot \frac{[\text{Drug}] \cdot K_b}{(1 + [\text{Drug}]\, K_b)}, \qquad (2)$$

where I_{site} is the intensity of the radioactive band as a percentage of the intensity of the band at a concentration where saturation was achieved I_{sat}, [Drug] is the concentration of NCS chrom and K_b is the binding constant for the site, where $K_b = 1/K_d$.

Fluorescence spectra were recorded on a Perkin Elmer 512 Spectrophotometer at ~2 °C. An MGW Lauda R20 constant temperature bath was used to control the temperature inside the cuvette. The excitation wavelength was 340 nm and emission spectra were taken between 360 and 600 nm. UV/Vis spectra were recorded on a dual beam 552A Perkin Elmer spectrophotometer. Binding curves of v/[DNA] versus v, were analyzed by linear fitting to the equation (3):[16]

$$\frac{v}{[\text{DNA}]} = \frac{1}{K_d} \cdot \frac{1}{(N - v)}, \qquad (3)$$

where v is the fraction of DNA sites occupied by molecules of drug, [DNA] is the concentration of oligonucleotide, K_d is the dissociation constant and N is the number of sites per oligonucleotide (N = 1). Fitting

of all the binding curves was conducted on a Macintosh II computer with the use of Kaleidagraph 2.1.4 program, Abelbeck software.

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