Covalent binding of antitumor benzoacronycines to double-stranded DNA induces helix opening and the formation of single-stranded DNA: Unique consequences of a novel DNA-bonding mechanism

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Abstract

The majority of DNA-binding small molecules known thus far stabilize duplex DNA against heat denaturation. A high, drug-induced increase in the melting temperature (Tm) of DNA is generally viewed as a good criterion to select DNA ligands and is a common feature of several anticancer drugs such as intercalators (e.g., anthracyclines) and alkylators (e.g., eteineascidin 743). The reverse situation (destabilization of DNA to facilitate its denaturation) may be an attractive option for the identification of therapeutic agents acting on the DNA structure. We have identified the tumor-active benzoacronycine derivative S23906-1 [([±])-cis-1,2-diacetoxy-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2,7-acridin-7-one] as a potent DNA alkylating agent endowed with a helicase-like activity. Using complementary molecular approaches, we show that covalent binding to DNA of the diacetate compound S23906-1 and its monooacetate analogue S28687-1 induces a marked destabilization of the double helix with the formation of alkylated ssDNA. The DNA-bonding properties and effects on DNA structure of a series of benzoacronycine derivatives, including the dicarbamate analogue S29385-1, were studied using complementory biochemical (electromobility shift assay, nuclease S1 mapping) and spectroscopic (fluorescence and Tm measurements) approaches. Alkylation of guanines in DNA by S28687-1 leads to a local denaturation of DNA, which becomes susceptible to cleavage by nuclease S1 and significantly decreases the Tm of DNA. The drug also directly alkylates single-strand DNA, but mass spectrometry experiments indicate that guanines in duplexes are largely preferred over single-stranded structures. This molecular study expands the repertoire of DNA-binding mechanisms and provides a new dimension for DNA recognition by small molecules. [Mol Cancer Ther 2005;4(1):71–80]
has led us to show that bonding of S23906-1 and congeners can be studied using complementary biochemical and spectroscopic approaches. The use of these methods allows us to delineate the structural perturbations of the DNA double helix induced by these benzoacronycines. This unprecedented helicase-like activity makes S23906-1 an atypical DNA-alkylating agent and it sets the stage for the design of a novel category of DNA binders acting as destabilizing agents. The unexpected effect of S23906-1 and congeners on DNA structure may well account for their potent antitumor effects.

### Materials and Methods

#### Chemicals and Biochemicals

The syntheses of the benzoacronycine derivatives have been reported previously (9, 14, 17, 18). To prepare the AT, IC, and GC ssDNA targets, the synthetic 30-mer oligonucleotides [d(TATATATATATAATATTTAATA-TATA), d(CGCGCGCGGGGGCGCGCGCCCGGGCGGCG- CG), and d(5'-CICICICICICICICICICICICICICICICICICICIC)] were 5'-end labeled using [γ-32P]ATP (3,000 Ci/mmol, Amersham, Little Chalfont, England) and polynucleotide kinase (Roche: Diagnostics, Meylan, France). The 20-bp ladder was obtained from Bio-Rad (Marnes-la-Coquette, France).

#### DNA Restriction Fragments

The XH-24- and 117-bp DNA fragments were obtained from the pBS plasmid digested with XbaI and HindIII or with EcoRI and PoulII restriction enzymes, respectively, in their respective digestion buffers. The digestion of the pTayD plasmid using EcoRI and HindIII restriction enzymes yields the 116-bp DNA fragment. The DNA fragments were then labeled at the HindIII or EcoRI sites with [α-32P]dATP (Amersham) and avian myeloblastosis virus reverse transcriptase (Ozyme, Saint Quentin Yvelines, France). Electrophoresis on a nondenaturing 10% (w/v) polyacrylamide gel served to remove excess radioactive nucleotide, with the desired 3'-end-labeled product being cut out of the gel and eluted overnight in 500 mmol/L ammonium acetate and 10 mmol/L magnesium acetate.

#### Gel Shift Studies

A typical alkylation reaction consisted of incubating 8 μL of radiolabeled DNA with 2 μL of buffer (10 mmol/L sodium cacodylate, pH 7.0) and 10 μL of the drug at the desired concentration for 1 or 15 hours in the dark at room temperature before adding 5 μL of a 50% glycerol solution containing tracking dyes. For the temperature-dependent studies of ssDNA alkylation, 50 μmol/L of S23906-1, S28687-1, or S23907-1 were incubated for 1 hour at 4°C, 20°C, 37°C, or 45°C. The DNA samples were resolved by electrophoresis under nondenaturing conditions in 6% or 20% polyacrylamide gels for about 5 hours at 300 V at room temperature in Tris-borate EDTA buffer (89 mmol/L Tris base, 89 mmol/L boric acid, 2.5 mmol/L Na2 EDTA, pH 8.3). Gels were transferred to Whatman No. 3MM paper, dried under vacuum at 80°C, and then analyzed on a 445SI PhosphorImager (Molecular Dynamics, Sunnyvale, CA).
Nuclease S1 Digestion

Samples (3 μL) of the radiolabeled 117-bp DNA fragment were incubated with S23687-1 at various concentrations from 1 to 50 μmol/L for 1 hour at 37°C in 10 μL of sodium cacodylate (1 mmol/L pH 7.0). After addition of the appropriate amount of the nuclease S1–specific buffer, the digestion was obtained by adding 4 units of S1 nuclease for a further 15 minutes incubation. Then 5 μL of native loading buffer containing tracking dyes were added and the samples were subjected to electrophoresis on a 6% native polyacrylamide gel for about 5 hours at 300 V at room temperature in Tris-borate EDTA buffer. The gel was then dried and analyzed on the Molecular Dynamics 445SI PhosphorImager.

Fluorescence Measurement of the Single-Strand/Double-Strand DNA Ratio

Briefly, double-strand calf thymus DNA (50 μmol/L) was incubated with or without increasing concentrations of S29385-1 (25, 50, or 100 μmol/L) for 16 hours at 37°C in 200 μL of a 1 mmol/L ammonium acetate buffer (pH 7.15) before the addition of a mixture of the picogreen dye (0.2 μL of the stock solution) and ethidium bromide (2.5 μmol/L). The fluorescence of both dyes was measured at an emission wavelength of 580 nm), which varies from 510 to 600 nm. The graph represents the maximum absolute S29385-1 (25, 50, or 100 μmol/L) was incubated with or without the double- or single-stranded DNA (50 μmol/L) for 16 hours at 37°C in 200 μL of a 1 mmol/L ammonium acetate buffer (pH 7.15) before the addition of a mixture of the picogreen dye (0.2 μL of the stock solution) and ethidium bromide (2.5 μmol/L). The fluorescence of both dyes was measured at an emission wavelength of 580 nm), which varies from 510 to 600 nm. The graph represents the maximum absolute

Thermal Denaturation Studies

The double-stranded XH-24-bp oligonucleotide (1.25 μmol/L oligonucleotide corresponding to 60 μmol/L nucleotides) was incubated alone (control Tm), with the various benzoacronycine derivatives (25 to 200 μmol/L) or with ET-743 or doxorubicin (50 μmol/L each) in 100 μL of 1 mmol/L ammonium acetate (pH 7.15) for 15 hours at 37°C. Samples were then ethanol precipitated and the pellet resuspended in 1 mL of BPE buffer (6 mmol/L Na2HPO4, 2 mmol/L NaH2PO4, 1 mmol/L EDTA, pH 7.1). The absorbance at 260 nm was measured every minute over the range 20°C to 90°C with an increment of 1°C/min, in 1-cm path-length quartz cells using a Uvikon 943 spectrophotometer thermostated with a Neslab RTE111 cryostat. Tm values were calculated from the midpoint of the first-derivative plots.

Alkylation of Oligonucleotides and Fluorescence Measurements

For the kinetic measurements, compound S28687-1 (5 μmol/L) was incubated with or without the double- or single-stranded AT, GC, or IC oligonucleotides (0.4 μmol/L) in 1 mL of the binding buffer containing 1 mmol/L sodium cacodylate (pH 7.0). The fluorescence of the compound was measured at different times after the addition of nucleic acid (excitation wavelength, 354 nm; emission wavelength, 510 nm).

Electrospray Ionization Mass Spectrometry

Oligodeoxynucleotides d(CGCGGGCCCGG, molecular weight = 3,648.4 Da), d(CGCGAATTCCGG, 3,646.4 Da), d(CGTTAAATTTACG, 3,644.4 Da), d(GGATGGATTGG, designated ssG, 3,765.5 Da), d(CCAATCCATTCC, designated ssC, 3,525.3 Da) were purchased from Eurogentec (Angleur, Belgium). The first three oligonucleotides form the self-complementary duplexes 1 to 3 respectively, and single strands ssG and ssC are mixed in equimolar ratio to obtain duplex 4. Oligonucleotide solutions were prepared in 100 mmol/L NH4OAc (pH 6.8). Ammonium acetate was chosen as the electrolyte for its compatibility with electrospray ionization mass spectrometry (ESI-MS). Solutions were heated up to 85°C for 5 minutes and cooled overnight to 20°C to form the desired duplex structures. Spectra of mixtures of DNA (5 μmol/L) + S28687-1 (10 μmol/L) were recorded. Methanol (15%) was added to the samples just before injection to obtain a stable electrospray signal. The rate of sample infusion into the mass spectrometer was 4 μL/min. Experiments were done on a Q-TOF Ultima Global mass spectrometer (Micromass, Manchester, United Kingdom) operated in the negative ion mode. The experimental settings are as follows: capillary voltage, −2.2 kV; source block temperature, 70°C; cone voltage, 35 V; RF lens 1 energy is optimized in each experiment (values between 17 and 40 V were used); source backing pressure, 2.9 mbar. Full-scan ESI-MS spectra were recorded in the m/z range 800 to 2,500, and 50 scans were summed for each spectrum. For the ESI-MS/MS spectra, the parent ion is selected in the first quadrupole and collided in the second quadrupole with argon (measured analyzer pressure, 3.0 × 10−5 mbar). As previously discussed (20), the relative intensities of the free and bound oligonucleotide observed in the full-scan ESI-MS spectra can be assumed to be proportional to the relative abundance of these species in solution. The concentration of bound ligand per DNA molecule (duplex or single strand) is obtained with the following equation:

\( A\text{[Bound ligand]} = C_0[I_{(1:1)} + 2I_{(2:1)}]/[I_{DNA} + I_{(1:1)} + I_{(2:1)}] \)

where C0 is the total DNA concentration, IDNA is the relative intensity of the DNA (duplex or single strand), and I(n:1) is the relative intensities of the complexes (n drug molecules bound to 1 DNA target) in the mass spectra.

Results

Gel Shift Analysis of the DNA Helix Destabilization Induced by S23906-1 and Benzoacronycine Derivatives

The first point arguing for an effect of some benzoacronycine derivatives on the destabilization of the DNA helix comes from structure-activity relationship studies in which we compared the ability of several benzoacronycine compounds (see structures in Fig. 1) to covalently bind DNA. The benzoacronycine derivatives used in this study include the lead compound diacetate S23906-1, monoacetate S28687-1, dicarbamate S29384-1, and asymmetric diester S28053-1, which all alkylate DNA, as well as
derivatives that do not form covalent complexes with DNA such as the diol S23907-1, the keto compound S28621-1, and benzoacronycine S23908-1. In the first instance, we used a 116-bp fragment as a substrate for alkylating drugs and compared the level of DNA retardation in polyacrylamide gel induced by the different benzoacronycine derivatives (Fig. 2). Identical experiments were done in parallel with ET-743 as a reference antitumor drug known to alkylate the N2 position of guanines (4) as does S23906-1 (8). We know from our previous studies that the gel shift reflects the covalent (bonding rather than binding) interaction of the drugs with DNA (8, 15–17). As shown in Fig. 2A, the effect of S23906-1 and some other benzoacronycine derivatives (S28687-1, S29385-1, and S28053-1) on the retardation of the 116-bp DNA migration is much more pronounced than that observed with ET-743, even though this latter molecule presents a significantly higher molecular weight (770.25 g/mol) than the benzoacronycine derivatives (from 447 g/mol for the monoacetylated compound S28687-1 to 561 g/mol for the monoacetylated monohemisuccinylated compound S28053-1). This observation provided the first argument to suggest that benzoacronycine derivatives induce an important structural change in the conformation of DNA to markedly modify its migration in polyacrylamide gels. In the course of these experiments, we observed that the dicarbamate compound S29385-1 (molecular weight = 547 g/mol; Fig. 1), which is the most efficient compound in the series at reducing the electrophoretic migration of the DNA, also induced the appearance of a DNA band that migrates faster than the substrate duplex DNA (see long horizontal arrow in Fig. 2A). The possibility that this compound induces DNA cleavage was rapidly eliminated (no cleavage activity was detected under denaturing conditions on sequencing gels) and then we were left with the hypothesis that this band corresponds to alkylated ssDNA. We then tested and validated this hypothesis through further gel experiments with DNA substrates of various length and biophysical measurements described below.

Experiments using different DNA fragments and short oligonucleotides suggest that the important level of gel retardation may be due to the destabilization of the DNA helix leading to the appearance of a band corresponding to alkylated ssDNA fragments. When using a small 24-bp DNA fragment (Fig. 2B), the gel shift experiments revealed that the DNA-S29385-1 covalent complexes are only present as an alkylated ssDNA form. With this compound, no more double-stranded alkylated DNA form could be detected, consistent with a pronounced drug-induced destabilization of the duplex DNA structure. In the same way, compound S28053-1, which does not induce the formation of fully ssDNA on the longer 116-bp fragment (Fig. 2A), induces denaturation of this short 24-bp DNA fragment (Fig. 2B). Increasing concentrations of the monacetate, diacetate, or dicarbamate compounds were used to compare their respective abilities to induce the formation of ssDNA. As shown in Fig. 2C, the opening of the DNA double helix is dependent on the drug concentration and the nature of the compound, with the dicarbamate S29385-1 being more potent than the monacetate S28687-1, which is itself more potent than the diacetate S23906-1 at inducing the opening of the DNA duplex. The dicarbamate molecule S29385-1 is extremely efficient at destabilizing DNA. This high reactivity is

Figure 2. Gel shift assay showing the formation of alkylated ssDNA by benzoacronycine derivatives. The various compounds (benzoacronycine derivatives or ET-743, 20 μmol/L each) were incubated for 24 h with the 116-bp radiolabeled DNA substrate (A) or the 24-bp DNA fragment (B). The level of migration of nonalkylated ssDNA was visualized in lane DNA/90°C where the labeled dsDNA was heated at 90°C before electrophoresis. C, increasing amounts of S28687-1, S23906-1, and S29385-1 were incubated overnight with the 24-bp DNA fragment for 24 h. Lane DNA, control DNA fragment alone; Lane Ladder, 20-bp DNA ladder. b, bound DNA fragments; f, free DNA fragments.
nuclease S1 major cleavage site.

b, 6% native gel. stranded positions by nuclease S1. The DNA fragments were resolved on a 1 h with the labeled 117-bp DNA fragment before the digestion at single-ssDNA cleavage by nuclease S1. S28687-1 was incubated for Figure 3.

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the pyrrolidizine alkaloid carmethizole (21). Gel-shifted reminiscent to that of bis(N-alkylcarbamate) derivatives of the pyrrolizidine alkaloid carmethizole (21). Gel-shifted dsDNA is hardly detected in the presence of S29385-1 (see the 20 μmol/L lane in Fig. 2C with this compound, with a 2:3 ratio of alkylated single-stranded to double-stranded DNA). However, the extent of gel shift varies from one DNA to another depending on the length and sequence. With another DNA fragment 117 bp in length, no alkylated ssDNA appeared in the presence of increasing amounts of S29385-1, but the gel retardation was very pronounced, suggesting that this DNA sequence is too long to allow a full-length destabilization of the double helix (data not shown).

Nuclease S1 Cleavage Experiments Reveal Local Opening of the DNA Helix

To further show that the benzoacronycines effectively induce a local opening of the DNA helix, the alkylated DNA was subjected to cleavage by the S1 nuclease, which is specific for ssDNA. If cleavage occurs, then it must reflect the drug-induced formation of single-stranded regions, that is, a local opening of the target duplex. In these experiments, a 117-bp DNA fragment was incubated in duplicate with increasing concentrations of compound S28687-1. Half of the samples were directly loaded onto the gel after the reaction and the other half were treated with nuclease S1 before the electrophoresis (Fig. 3). Gel retardation occurs in the nuclease-free samples, thus reflecting the efficient alkylating properties of S28687-1. In contrast, in the nuclease-treated samples (4 units of nuclease S1 for 15 minutes), we detected a significant degradation of the DNA, consistent with the suspected opening of the drug-induced DNA helix. It is interesting to note that the S1 cleavage essentially occurs at a well-defined site (see arrow marked N in Fig. 3), which may correspond to a major site of local destabilization of the double helix of DNA induced by the benzoacronycine derivative. The local opening of the helix must be large to allow the correct positioning of the S1 nuclease and subsequent cleavage at the ssDNA site.

A concentration-dependent effect is also shown in Fig. 4 with S28687-1. In this case, the DNA samples were electrophoresed on a polacrylamide gel under denaturing conditions (sequencing-type gel). Here again, prominent sites of cleavage by S1 nuclease can be detected with the drug. The gel shift of the DNA material reflects the alkylation process.

Fluorescence Measurements to Identify the Formation of ssDNA

Fluorescence measurements were carried out to measure the variation of single-to-double-stranded DNA ratio using a mixture of picogreen and ethidium bromide dyes, according to a recently described method (19). Picogreen is considered specific for dsDNA, whereas ethidium bromide binds equally well to both single- and double-stranded DNA. Double-stranded calf thymus DNA (which is available in large quantities) was incubated with increasing concentrations of S29385-1 (and other benzoacronycines) for 16 hours before an ethanol precipitation to remove unbound molecules. A mixture of picogreen and ethidium bromide was then added to the DNA sample and the fluorescence emission was measured under an excitation at 503 nm. The graph in Fig. 5 represents the ratio of picogreen fluorescence (525 nm) over the ethidium bromide fluorescence (580 nm). The diol compound S23907-1, which does not react covalently with DNA, has practically no effect on the fluorescence of the dyes. In contrast, S29385-1, S23906-1, and S28687-1 induce a large decrease of the picogreen fluorescence without affecting the ethidium bromide fluorescence and again this is consistent with the formation of ssDNA upon drug treatment. Therefore, this fluorescence approach confirms the drug-induced local opening of the DNA helix.

Thermal Denaturation Studies

Finally, as the last method to investigate the opening of the DNA induced by the benzoacronycine compounds, we resorted to melting temperature measurements using the same 24-bp oligonucleotide as used in the gel shift experiments. The duplex oligonucleotide was reacted with the test compounds and then slowly heated up to 90°C to denature it. The melting temperature of the free duplex was then compared with those measured with the drug-treated samples. The benzoacronycine compounds were tested at different concentrations (Fig. 6A) and parallel experiments were done with ET-743 and the anticancer drug doxorubicin, a well known anthracycline that does not alkylate DNA but strongly binds to DNA by intercalation between base pairs (Fig. 6B). The melting
temperature of the DNA increases with doxorubicin and ET-743, whereas it decreases with the benzoacronycines. The benzoacronycine derivatives that alkylate DNA (S23906-1, S28687-1, and S29385-1, but not the inactive compound S23907-1) significantly lower the $T_m$ of the oligonucleotide. The negative $\Delta T_m$ values reflect the drug-induced destabilization of the DNA double helix, in sharp contrast to the positive $\Delta T_m$ values obtained with ET-743 and doxorubicin, which, unlike the benzoacronycine compounds, both reinforce the cohesion between the two strands of the DNA.

Altogether, our different complementary approaches using gel shift, S1 nuclease cleavage, fluorescence, and melting temperature clearly establish that covalent binding of the benzoacronycines to DNA results in a marked destabilization of the DNA with the appearance of single-stranded regions. Subsequently, this helicase-like activity prompted us to investigate whether these compounds could directly alkylate ssDNA.

**Bonding of S23906-1 and Benzoacronycine Derivatives to ssDNA**

We first used gel shift experiments to test the ability of the diacetate S23906-1, the monoacetate S28687-1, or the diol S23907-1 compounds (50 μmol/L each) to alkylate single-stranded oligonucleotides containing only GC, AT, or IC base pairs. These simple oligonucleotide models have been used previously to determine the specificity of S23906-1 for the 2-amino group of guanines in duplex DNA (8). Gels in Fig. 7A clearly identify S23906-1 and S28687-1 as efficient alkylating drugs specifically interacting with GC-containing single-stranded oligonucleotides but not AT (data not shown) or IC base pairs. In contrast, the diol derivative is unable to covalently link GC, AT, or IC base pairs. This result correlates with previously published data using double-stranded oligonucleotides (8) and indicates that the specificity of S23906-1 for the NH$_2$ exocyclic group of the guanine residues is preserved with ssDNA. The reactivity of the different compounds toward ssDNA is identical to what we had seen previously with duplex DNA: (a) the diol S23907-1 fails to alkylate DNA, be it a single- or a double-stranded substrate, (b) the acetylated compounds S23906-1 and

**Figure 4.** ssDNA cleavage by nuclease S1 in the presence of S28687-1. S28687-1 was incubated for 1 h with the labeled 117-bp DNA fragment before the digestion at single-stranded positions by nuclease S1. The DNA fragments were resolved on a 8% polyacrylamide gel under denaturing conditions (8 mol/L urea). Arrows point to the drug-induced S1 nuclease cleavage sites. G, dimethylsulfate-piperidine markers specific for guanines.

**Figure 5.** Fluorescence intensity of the single-to-double-stranded DNA ratio in the presence of graded concentrations of S29385-1 (■), S23906-1 (□), S28687-1 (▲), or S23907-1 (○). Double-stranded calf thymus DNA was incubated with increasing concentrations of the benzoacronycine derivatives for 16 h at 37°C before the addition of the mixture of picogreen and ethidium bromide dyes as described in Materials and Methods. Mean values of three independent experiments.
S28687-1 are both able to alkylate double- as well as ssDNA, and (c) the monoacetate form S28687-1 is more efficient than the diacetate S23906-1 to alkylate ssDNA as it does for dsDNA. The alkylation of ssDNA by S23906-1 and S28687-1 is a temperature-dependent process (data not shown). The level of gel retardation of the DNA by the di- or monoacetylated compounds becomes more important when the temperature is raised from 4°C to 45°C. This is again entirely consistent with our previous findings with duplex DNA (8).

Finally, the G-specific single-strand reactivity of the studied compounds was investigated by fluorescence spectroscopy using the intrinsic fluorescence of the benzoacronycine chromophore and the GC, IC, and AT oligonucleotides as substrates. The kinetics of the reactions between S28687-1 and the various oligonucleotides, either in their double-stranded (Fig. 7B) or single-stranded (Fig. 7C) forms, were followed by measuring the fluorescence emission in the 500- to 550-nm range upon excitation at 354 nm. The addition of double- or single-stranded GC oligonucleotides induces a marked time-dependent quenching of the fluorescence of the drug, whereas no variation of the fluorescence was observed in the presence of S2687-1.

Figure 6. Effects of the benzoacronycine derivatives on the thermal denaturation of DNA. A, increasing concentrations of diacetate (S23906-1), monoacetate (S28687-1), diol (S23907-1), or dicarbamate (S29385-1) compounds were incubated with the double-stranded 24-bp oligonucleotide in BPE buffer and the absorbance at 260 nm was measured every minute over the range 20°C to 90°C with an increment of 1°C/min. The ΔTm values correspond to the difference between the midpoint of the first-derivative plots obtained in samples containing both DNA and the various tested compounds minus that obtained in samples containing DNA alone as a control. B, comparison of the effect of benzoacronycine derivatives (200 μmol/L), ET-743 (50 μmol/L), or doxorubicin (DOXO; 50 μmol/L) on the denaturation of the double-stranded 24-bp DNA.

Figure 7. Bonding of the benzoacronycine derivatives to ssDNA. A, increasing concentrations of S23906-1, S28687-1 or S23907-1 were incubated with 5’-labeled single-stranded GC (top) or IC (bottom) containing oligonucleotides in cacodylate buffer for 16 h before electrophoresis on a 10% native polyacrylamide gel. Fluorescence measurements to evaluate the kinetics of binding of S28687-1 to single- versus double-stranded DNA. S28687-1 (5 μmol/L) was incubated alone (○) with double-stranded (B) or single-stranded (C) AT (△), GC (●) or IC (□) containing oligonucleotides (0.4 μmol/L) in 1 mL of sodium cacodylate buffer. The intrinsic fluorescence of the compound was measured from 5 min to 2 h after the addition of nucleic acid (excitation wavelength, 354 nm; emission wavelength, 510 nm). The graphs are presented as the percentage of decrease of the fluorescence due to the DNA binding.
of the IC- or AT-containing oligonucleotides. This
G-specific reaction confirms that the covalent bonding
involves the exocyclic amino group of the guanine base. As
judged from the magnitude of the quenching, it seems that
both double and single G-containing DNA provide
suitable, equally strong substrates for benzoacronycines.

**DNA Duplex Preference**

The above experiments indicate that S28687-1 can
alkylate both single-stranded and double-stranded DNA
but mass spectrometry measurements indicated that
duplex DNA is largely preferred over ssDNA. The
compound was incubated for 24 hours with different
oligonucleotide sequences and the fraction of ligand
bound to each oligonucleotide was determined by mass
spectrometry. Figure 8 shows that binding of S28687-1
to duplex DNA is largely preferred over single-strand DNA.
The amount of drug molecules bound to the single-
stranded oligonucleotide ssG (binding to ssC is negligible)
is >10 times lower than to the equivalent duplex 4. Binding
to three other duplexes was also considerably higher than
to ssG. The helical structure of duplex DNA is not
absolutely required but it greatly facilitates efficient
bonding of the benzoacronycine compound to DNA.

A complementary experiment termed “collisional acti-
vation” was done on the covalent adduct of S28687-1
bound to duplex 4 (data not shown). This ESI-MS/MS
experiment shows that the complex (m/z 1,534.6) disso-
ciates into the single strands, with the drug remaining
attached exclusively on the G-rich strand ssG. The guanine
bases are the preferred sites for the covalent attachment
of the benzoacronycine compound and this is in perfect
agreement with the biochemical data.

**Discussion**

S23906-1 is the leading compound of the benzoacronycine
series, developed as an antitumor agent (11, 17). This
compound has shown remarkable *in vivo* antitumor activity
in different models in mice (10) and it is currently
undergoing phase I clinical trial. Its antitumor activity is
undoubtedly linked to its DNA-binding capacity (14), but
the reasons why this compound is so active, compared with
other conventional alkylating drugs, remain an open
question. We suspected that the drug might exert an
uncommon, specific effect on DNA to provoke a distinctly
lethal type of lesion. The data reported here show that
S23906-1 induces the appearance of ssDNA after alkylation.
It will be important to determine how the DNA lesions
induced by S23906-1 are repaired, through which repair
system, and with what lethal consequences for cells.

Although the exact mode of binding of S23906-1 to DNA
remains unclear (no structural data are available as yet), we
have previously showed that this compound does not
intercalate into DNA and specifically reacts with the N2
position of guanines in the minor groove of duplex DNA (8).
Here we have used a series of benzoacronycine analogues
to improve our knowledge of the mode of binding of
S23906-1 to DNA. Using complementary approaches, we
show that the alkylation of DNA by these compounds leads
to a marked destabilization of the DNA double helix with
the formation of alkylated ssDNA. The gel shift assays
indicate very clearly that S23906-1 and its dicarbamate
analogue S29385-1 behave quite distinctly from the drug
ET-743, known as a G-N2 alkylating agent (5). The marked
difference between these two categories of drugs was also
revealed in the melting temperature measurements. ET-743
potently stabilizes duplex DNA against heat denaturation,
as is the case for the intercalating drug doxorubicin,
whereas S23906-1 and S29385-1 both markedly destabilize
the duplex. The fluorescence measurements also speak of
the appearance of drug-induced, alkylated ssDNA, which
can be specifically cleaved by the S1 nuclease. Altogether,
these experiments combining biochemical and physico-
chemical approaches establish the unique property of
S23906-1 and its dicarbamate analogue, which exhibit a
helicase-like activity. As far as we are aware, this action
is unique to this class of compounds and has not been
reported previously with other alkylating drugs. It is
therefore tempting to conclude that this property is
implicated in if not partially responsible for the anti-
tumor activity of S23906-1. We have recently presented
evidence for a correlation between the cytotoxicity of
the benzoacronycines and their capacity to alkylate DNA
(14). The most cytotoxic compounds are also the most
efficient at destabilizing DNA. For example, S29385-1 is
more cytotoxic than S23906-1 *in vitro*, possibly because of
its superior reactivity toward nucleophiles. We are
currently conducting additional experiments at the cell
level to determine if the helicase activity of S23906-1
evidenced here at the molecular level with purified
substrates also occurs in cells. Electron microscopy
observation of drug-DNA genomic DNA might provide
an answer to this question.

In terms of structure-activity relationships, the present
data fully confirm our previous observations. The diol
compound and the compounds bearing a 1-2 double bond
(benzoacronycine S23908-1) or a keto group at position 1

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**Figure 8.** Amount of S28687-1 bound (µmol/L) to each DNA sequence (base pairs or nucleotides) determined by ESI-MS. S28687-1 (10 µmol/L) was incubated with each DNA duplex or single strand (5 µmol/L) and mass spectra were recorded after 24 h incubation at 20°C. The concentration of covalently bound ligand was calculated with Eq. A (see Materials and Methods). Duplex 4 was made from annealing oligonucleotides ssG and ssC.
(S28621-1) are all inert toward DNA and considerably less cytotoxic than the DNA reactive analogues (17). The notable exception is the cyclic carbonate derivative S23905-1, which fails to react with DNA in vitro but is highly cytotoxic, presumably due to a metabolic activation in cells into a reactive ester form, as previously discussed (9, 14).

S23905-1 and related benzoacronycines induce a massive distortion of DNA leading to the opening of the double helix. Although the exact reaction scheme is not yet elucidated, our current knowledge of the structure/DNA-binding relationships (including recently published mechanistic information, ref. 15) suggests that there is a two-step process: an essential alkylation of G-N2 followed by a structural deformation of the DNA helix, possibly due to the planar ring system of the molecule. This strand invasion effect is unique for a small molecule. It is known with nucleic acid mimics, PNA in particular (22). We are not aware of any other small molecule that exerts this helicase-type effect, which is energy consuming. Under physiologic conditions, the two strands of a DNA helix are closely associated and an important energy must be provided to disrupt (invade) the helix (23). A distinct but comparable effect has been reported with the DNA intercalating antitumor drug actinomycin, which can disrupt the Watson-Crick base pairing at a 5'-GXC/CYG-5' intercalation site, with the looped-out base interacting perpendicularly with the stacked base-drug complex (24).

A similar minor groove guanine-flipping process may occur with S23906-1 upon alkylation of DNA. Actinomycin strongly binds to GpC-containing duplex DNA, but recent studies have shown that this drug can also interact with ssDNA devoid of GpC sites (25, 26). This situation is reminiscent of that described here with S23906-1, which binds to guanine residues in duplex DNA, to a lower extent to ssDNA, and causes major DNA distortions. The finding that benzoacronycine derivatives can open the DNA duplex may have important biological implications on understanding the anticancer activities of this drug. We can hypothesize that S23906-1 promotes the activity of enzymes acting on ssDNA, such as topoisomerase III. The present study enlarges the repertoire for DNA recognition by small molecules and opens novel routes to design other anticancer agents.

As a concluding remark, this study shows that our perception of DNA recognition by small molecules needs to be reconsidered to include DNA-destabilizing agents. For decades, since the discovery of the intercalation model for acridines in the early 1960s (27), the search for DNA-targeted antitumor agents has been focused on molecules that stabilize duplex (and more recently triplex and quadruplex) DNA. Intercalation and/or classic alkylation with increased $T_m$ offer a facile view to block replication and transcription machineries through the perturbation of DNA structure processing enzymes such as topoisomerases. Little attention was paid to molecules acting via the opposite process of DNA structure opening. Local opening of the structure of DNA is known to occur with certain proteins but this is an idea more difficult to envision with small molecules, which usually do not cover more than 2 to 4 bp. This prospect is now open to experimental testing.

References


Covalent binding of antitumor benzoacronycines to double-stranded DNA induces helix opening and the formation of single-stranded DNA: Unique consequences of a novel DNA-bonding mechanism


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