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DNA-Binding Properties of Cosmomycin D, an Anthracycline with Two Trisaccharide Chains

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Cosmomycin D (CosD) is the major constituent fraction isolated from a culture of *Streptomyces olindensis* ICB20. The ability of this compound to intercalate with double-stranded DNA was studied by gel mobility shift assays and electrospray ionization mass spectrometry (ESI-MS). ESI-MS experiments showed that the complex of CosD with 16-mer double-stranded DNA was at least as stable as a complex of daunorubicin with the same DNA sequence. This is the first study showing DNA binding properties of an anthracycline containing a β-rhodomycinone aglycone chromophore *O*-linked to two trisaccharide chains.

Anthracyclines are an important family of natural products produced by microorganisms of the actinomycetes group. They are tetracyclic aromatic polyketides which share a 7,8,9,10-tetrahydrotetracene-5,12-quinone structure. Some members of the anthracycline group of antibiotics show interesting pharmaceutical, mostly anticancer, activities[1,2]. Anthracyclines that are used for cancer treatment include daunorubicin (Fig. 1A; DAU; daunomycin) and its hydroxylated derivative doxorubicin (Fig. 1B; DOX; adriamycin). Other clinically useful natural anthracyclines are aclacinomycin A and nogalamycin, synthesized by *S. galilaeus* and *S. nogalater*, respectively[3,4].

The biological activities of the anthracyclines are proposed to be the result of drug-induced damage or structural alterations to DNA. This damage is mediated by quinone-generated radicals, stabilization of a cleavable complex formed between DNA and topoisomerase II, or intercalation-induced distortion of the double helix[5]. The anthracyclines are thought to intercalate between DNA base pairs forming a DNA-drug-topoisomerase II complex[6]. Interactions of anthracyclines with DNA are more complex than for simple intercalators such as ethidium bromide. For example, daunorubicin has three functional domains: the intercalating anthraquinone ring system; the daunosamine sugar which interacts with the minor groove; and substituents on the A-ring which hydrogen bond with DNA bases[7].

As a consequence of the interaction of the anthracycline sugars and oligosaccharides with DNA, their identities have implications in the structures of complexes with DNA and in the biological activities of the anthracyclines[8,9]. In the anthracycline family the polyketide-derived aglycones are glycosylated with a wide array of different sugars either as single sugar moieties or sugar side chains. These sugars are 6-deoxysugars and generally comprise an aminosugar, such as L-daunosamine, L-rhodosamine or nogalamine. Some other deoxysugars such as 2-deoxy-L-fucose, L-cinerulose, L-rhodinose or L-nogalose can also be found. In addition, some anthracyclines contain acetal moieties possibly formed by cleavage of sugars.

In the present work, two approaches were used for rapid, sensitive screening of *in vitro* interactions of CosD (Fig. 1C)[10,11] with dsDNA. These were gel mobility shift assays[12] and electrospray ionization mass spectrometry

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(ESI-MS). This is the first study investigating the DNA binding properties of an anthracycline containing a β-rhodomycinone aglycone *O*-linked to two trisaccharide chains and the first study that compares the anthracycline DNA-binding using gel mobility shift assays and ESI-MS.

Material and Methods

Cell Culture

Mycelia of *Streptomyces olindensis* ICB20 were grown in 100 ml of TSB medium (Difco) for 24 hours at 30°C with shaking (250 rpm). The seed cultures were used to inoculate (2% v/v) 50 ml of R5M medium. R5M is a modification of R5 medium[13] and contains the following components: glucose (10 g/liter), yeast extract (Difco; 5 g/liter), casamino acids (0.1 g/liter), and $MgCl_2 \cdot 6H_2O$ (10.1 g/liter) adjusted to pH 7.3 before autoclaving. After autoclaving 2.5 ml of 0.5% (w/v) KH_2PO_4, one ml of 5 M $CaCl_2$ and 2 ml of R5 trace elements solution were added aseptically. Cultures in R5M were incubated for 48 hours at 30°C with shaking (250 rpm).

Purification of the CosD from *S. olidensis* ICB20

This procedure was necessary to the anthracycline CosD is not available commercially. The *S. olindensis* ICB20 cultures were centrifuged, the pellets were discarded and the supernatants were applied to a solid-phase extraction cartridge (Sep-Pak Vac; 10 g; Waters). The cartridges were eluted using a linear 0 to 66% acetonitrile gradient in 50 mM potassium phosphate buffer pH 6.5, over 40 minutes at a flow rate of 10 ml/minute. Fractions were taken every 2.5 minutes and analyzed by HPLC as previously described,[14] except that the wavelength used for detection was 488 nm. Anthracyclines were found in seven fractions collected from 20 to 37.5 minutes. Five of these fractions were purified further, and yields (per liter of culture) were: 37.6 mg (CosD), 8.1 mg (fraction 3), 11.5 mg (fraction 4), 11.6 mg (fraction 5), and 11.8 mg (fraction 7). These were dried *in vacuo* and redissolved in a small volume of methanol : DMSO (1 : 1). CosD was purified further by semipreparative HPLC, using an XTerra RP18 (7.8× 300 mm; Waters) column and mixtures of acetonitrile and 0.1% trifluoroacetic acid in water as the mobile phase. Isocratic conditions and a flow rate of 3 ml/minute were used in all cases. CosD was purified using 30% acetonitrile. It was collected in bottle containing 0.1 M potassium phosphate buffer, pH 7.0. These solutions were diluted 4-fold with water, applied to a solid-phase extraction cartridge (Sep-Pak Vac; 500 mg; Waters), washed with water to eliminate salts and dried *in vacuo*.

Gel Mobility Shift Assays

Gel mobility shift assays were carried out by the method previously described[12].

ESI-MS of Anthracycline dsDNA Complexes

All reagents used were of the highest grade commercially available. MilliQ water (Millipore™) was used in all experiments. Oligonucleotides were obtained from Geneworks (South Australia) and purified as previously described[15]. Oligonucleotide concentrations were calculated using values for molar absorption coefficients from the web site Oligonucleotide Properties Calculator[16]. Complementary DNA strands used in these experiments were 5′-d(GATCGAATCGGACGAG)-3′ and 5′-d(CTCGTCCGATTCGATC)-3′. The strands were stored in water at a concentration of 1 mM. The strands were prepared for annealing by combining them in a 1 : 1 ratio and freeze-drying using a Savant SpeedVac. The dried DNA strands were redissolved in 0.1 M ammonium acetate, pH 8.5 to give a final concentration of 1 mM for dsDNA. The strands were heated in a water bath to 70°C for 15 minutes and allowed to cool to room temperature overnight.

Treatment of dsDNA with Anthracyclines: Appropriate volumes (0.5～5 μl) of fraction CosD (1 mM in 50% methanol in H_2O) or daunorubicin (1 mM in H_2O) were pipetted into Eppendorf tubes and freeze-dried using a Savant SpeedVac. dsDNA (1 μl) was added so that the final concentration of DNA in all experiments was 20 μM and the concentration of COSD or DAU were in the range 10～180 μM. The final volume (50 μl) was made up with 0.1 M ammonium acetate, pH 8.5, and the mixtures were equilibrated on ice at 4°C for 30 minutes. Samples were diluted 2-fold using 0.1 M ammonium acetate, pH 8.5 for ESI-MS analysis.

ESI-MS Analysis: All mass spectra were acquired using a Micromass (Wyntheshawe, UK) Qtof2 mass spectrometer with a Z-spray probe. The mass analyzer has an *m*/*z* range of 10000. Samples were injected directly using Harvard Model 22 syringe pump (Natick, MA, USA) at a flow rate of 10 μl minute^{-1}. Negative ion ESI mass spectra were acquired using a probe tip potential of 2600 V, a cone voltage of 50 V, and the source block and desolvation temperatures were 40°C. The transport and aperture were set to 2.0 and 13.0, respectively. In most experiments, spectra were acquired over the range *m*/*z* 400～3000. Typically, 50 to 70 scans were summed to obtain representative spectra. The data were calibrated against a standard CsI solution (750 μM) over the same *m*/*z* range.

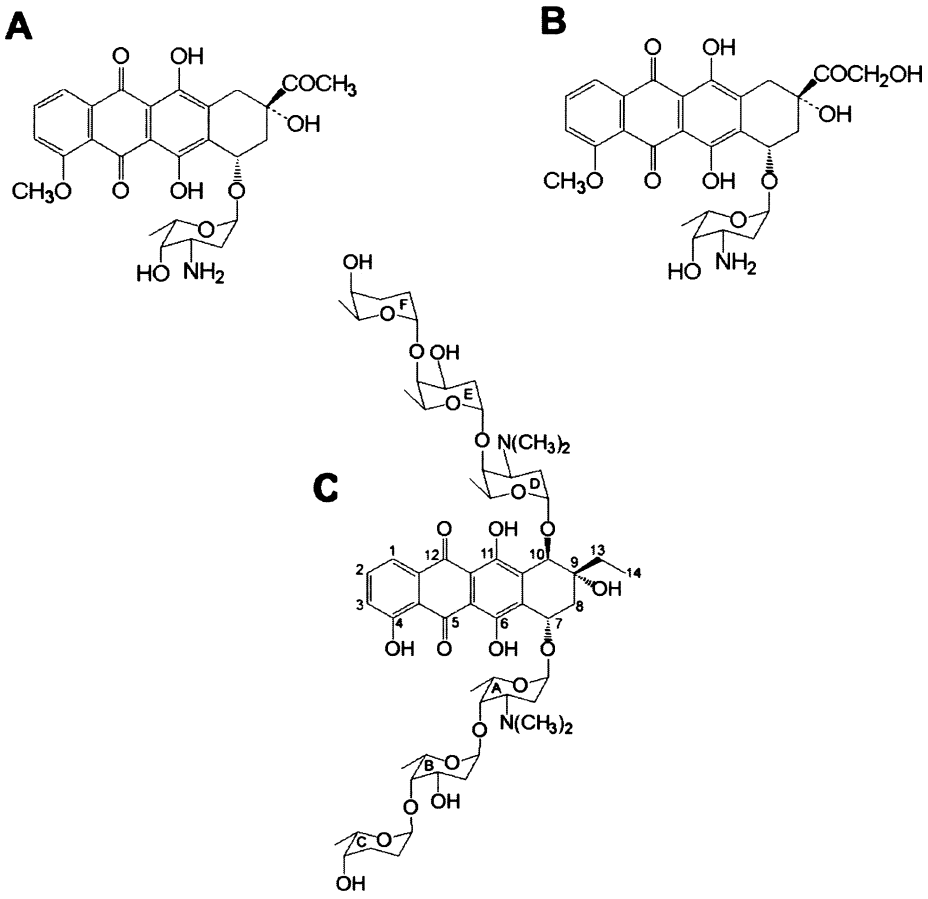
NMR Spectroscopy

CosD was dissolved in CD_2Cl_2 to carry out 1D (^1H-, ^{13}C NMR) as well as homo- and heteronuclear 2D NMR experiments (H,H-COSY, HSQC and HMBC) using Varian Inova 300 (operating field strength 7.05 T) and 400 (operating field strength 9.4 T) instruments, respectively.

^1H NMR (400 MHz, CD_2Cl_2, δ): 13.78 (br s, 11-OH), 12.86 (br s, 4-OH), 12.16 (br s, 6-OH), 7.91 (dd, 7.7, 1.8 Hz, 1-H), 7.74 (dd, 8.8, 7.7 Hz, 2-H), 7.33 (dd, 8.8, 1.8 Hz, 3-H), 5.45 (br d, 4 Hz, 1A-H), 5.36 (br d, 4 Hz, 1D-H), 5.15 (dd, 4, 1.6 Hz, 7-H), 4.90 (br d, 4 Hz, 1B-H), 4.96 (d, 1 Hz, 10-H), 4.87 (br s, 1C-H), 4.81 (br s, 1F-H), 4.77 (br d, 4 Hz, 1E-H), 4.51 (dq, 6.4, 2 Hz, 5B-H), 4.39 (dq, 6.4, 2 Hz, 5E-H), 4.17 (dq, 6.4, 2 Hz, 5A-H), 4.02 (dq, 6.4, 2 Hz, 5D-H), 3.97 (dq, 6.4, 2 Hz, 5C-H and 5F-H), 3.86 (dd, 10, 2 Hz, 4B-H and 4E-H), 3.72 (br s, 4A-H and 4D-H), 3.63 (br s, 3B-H and 3E-H), 3.53 (br s, 4C-H and 4F-H), 3.41 (br s, 3A-H and 3D-H), 2.28 (dd, 16, 5 Hz, 8-Heq), 2.15 (s, N-CH_3), 2.2～1.9 (m, complex, methylene protons), 1.9～1.5 (m, complex, methylene protons), 1.25 (d, 6.4 Hz, 6A-H_3), 1.20 (d, 6.4 Hz, 6D-H_3), 1.18 (d, 6.4 Hz, 6B-H_3), 1.09 (d, 6.4 Hz, 6E-H_3), 1.08 (d, 6.4 Hz, 6C-H_3), 1.06 (t 7.8 Hz, 14-H_3) and 1.01 (d, 6.4 Hz, 6F-H_3) ppm.

^{13}C NMR (75 MHz, CD_2Cl_2, δ): 190.8 (C-5), 186.5 (C-12), 162.9 (C-4), 158.1 (C-11), 157.9 (C-6), 138.9 (C-10a), 138.1 (C-12a), 137.8 (C-2), 134.1 (C-6a), 125.2 (C-3), 120.0 (C-1), 116.8 (C-4a), 112.6 (C-5a), 112.5 (C-11a), 102.8 (C-1A), 100.6 (C-1C), 100.6 (C-1F), 100.4 (C-1B), 100.3 (C-1E), 97.4 (C-1D), 82.7 (C-4B), 82.6 (C-4E), 75.0 (C-4A), 74.9 (C-4D), 72.1 (C-9), 72.0 (C-7), 70.5 (C-10), 69.5 (C-5C), 69.2 (C-5F), 69.1 (C-5A and C-5D), 68.1 (C-5B), 68.0 (C-5E), 67.3 (C-4C), 67.3 (C-4C and C-4F), 66.3 (C-3B and C-3E), 62.6 (C-3A), 62.5 (C-3D), 44.1 (N-CH_3), 43.9 (N-CH_3), 35.4 (C-2B), 34.7 (C-2E), 32.3 (C-8), 31.0 (C-13), 30.7 (C-2A), 30.5 (C-2D), 27.1 (C-3C and C-3F), 25.3 (C-2C and C-2F), 18.8 (C-6D), 18.6 (C-6A), 18.2 (C-6B and C-6E), 18.1 (C-6C and C-6F), 7.6 (C-14) ppm.

Fig. 1.



A. Structure of danuorubicin (daunomycin). B. Structure of doxorubicin (adriamycin), and C. Structure of CosD purified from *Streptomyces olindensis* ICB20.

Results

Purification and Characterization of Cosmomycin D

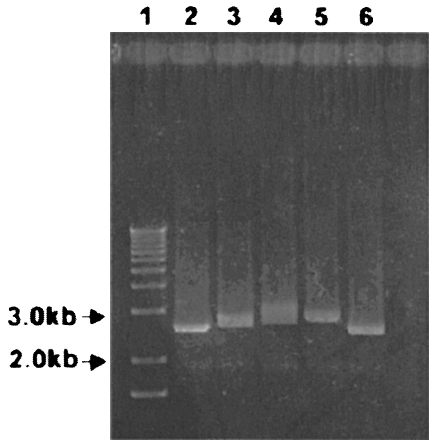
Supernatants from *S. olindensis* ICB20 cultures were fractionated by HPLC, giving seven fractions. An ESI mass spectrum (data no show) showed that one fraction contained a single compound giving an ion $[COS+H]^+$ (*m*/*z* 1189.9; mass of CosD 1188.9 Da) in close agreement with the mass expected from the structure shown in Fig. 1C (1188.6 Da). Tandem mass spectrometry (ESI-MS/MS) analysis of the purified fraction showed two losses of 244.2; as the collision energy was increased there was a further loss of 157.1. This fragmentation pattern is consistent with the presence of two trisaccharide chains each consisting of a terminal L-rhodinose sugar, attached to a 2-deoxy-L-fucose residue, and an L-rhodosamine residue attached to the β-rhodomycinone ring of CosD. The glycosidic bond between the two terminal residues (rhodinose and deoxyfucose) and the rhodosamine sugar of each trisaccharide are the most susceptible to fragmentation, followed by loss of one of the remaining rhodosamine residues. It is likely that protonation of the tertiary amine of the rhodosamine residue directs fragmentation at the adjacent glycosidic linkage.

While the ^{1}H NMR data confirmed the identity of the sugar moieties as l-rhodosamine, 2-deoxy-L-fucose and L-rhodinose, observed $^3J_{C\text{-}H}$ long-range couplings in the HMBC spectrum determined the bonds connecting the saccharide chain, and the bonds between the trisaccharide chains and the β-rhodomycinone aglycone (1A-H couples with C-7; 1D-H couples with C-10).

Gel Mobility Shift Assays

In previous work, we showed that anthracyclines bound plasmid DNA and retarded its migration through an agarose gel[17]. In the present work, CosD was tested in the same way. Fig. 2 compares the migration in an agarose gel of complexes of plasmid DNA with daunorubicin, doxorubicin and the CosD itself. The gel mobility shifts (based on the mobility of linear DNA) relative to anthracycline-free DNA plasmid and for mixtures of DOX, DAU or CosD (all at 1 ng/μl) with plasmid DNA was 0.1～0.2 (for DOX and DAU), and 0.4 kb for CosD. The concentrations of DAU, DOX and CosD in the assays were 1.9 μM, 1.8 μM and 0.9 μM, respectively. Clearly, CosD has the greatest effect on mobility of DNA through the gel. Decreased gel mobility represents a change in DNA structure to a more open form. DOX and DAU are both known to bind to DNA by intercalation[18,19]. Intercalation between DNA base pairs lengthens dsDNA, retarding its migration through the gel[20].

Fig. 2. Gel mobility shift assay comparing migration of complexes of DAU, DOX or CosD with plasmid DNA.



1. Standard DNA ladder. 2. Control pUC18. 3. DAU (1 ng/μl; 1.9 μM). 4. DOX (1 ng/μl; 1.8 μM). 5. CosD (1 ng/μl; 0.9 μM). 6. Control pUC18.

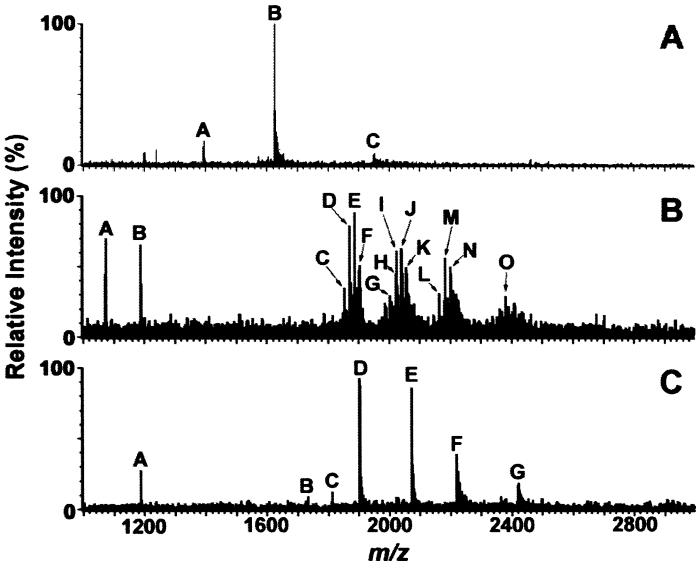
ESI-MS of Anthracycline-dsDNA Complexes

We have previously investigated the application of ESI-MS for examining the interactions of the extensively studied anthracyclines, daunorubicin and nogalamycin with double-stranded (ds) DNA, but the present work is the first demonstration of the technique for studying anthracyclines with the glycosylation pattern exhibited by CosD.

Fraction CosD was chosen for ESI-MS analysis of its interaction with 16 mer dsDNA because it showed the greatest effect on mobility of dsDNA. In previous experiments we examined the interaction of daunorubicin (daunomycin) with self-complementary 16 mer dsDNA[15]. Ambiguities arose in analysis of spectra since ions with an even number of charges arising from dsDNA-drug complexes containing an even number of drug molecules have the same *m*/*z* as ions from ssDNA with half the number of charges and half the number of drug molecules bound. In subsequent studies[21] and in the present work we have used non-self-complementary dsDNA to avoid this problem with analyses.

In a preliminary experiment, an anthracycline-dsDNA complex was prepared by adding fraction CosD to unannealed DNA (6 : 1) and then subjecting the mixture to

Fig. 3. Comparison of negative ion ESI mass spectra of dsDNA, dsDNA annealed in the presence of CosD, and dsDNA treated with CosD after annealing.



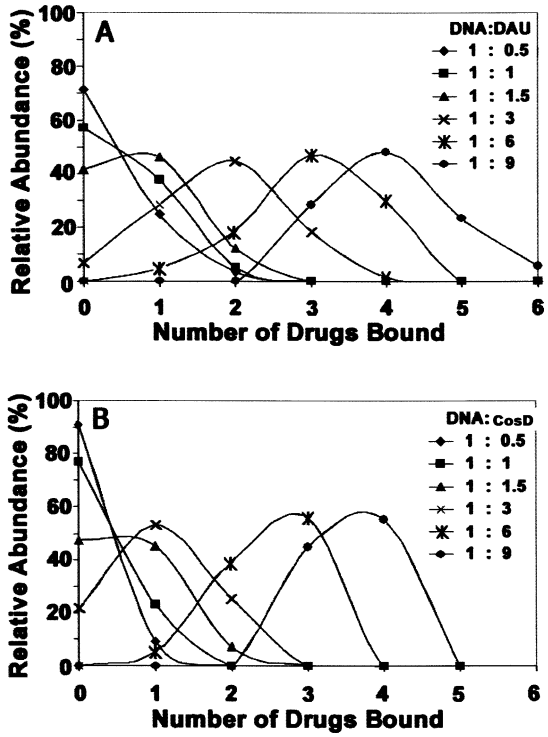
A. ESI mass spectrum of d(GATCGAATCGGACGAG). (CTCGTCCGATTCGATC). B. ESI mass spectrum of d(GATCGAATCGGACGAG). (CTCGTCCGATTCGATC) annealed in the presence of CosD. C. ESI mass spectrum of d(GATCGAATCGGACGAG). (CTCGTCCGATTCGATC) treated with CosD after annealing.

an annealing step (heating to 70°C and allowing to cool slowly to room temperature). Fig. 3 shows negative ion ESI mass spectra of dsDNA alone (A) and dsDNA annealed in the presence of CosD (B). The ions at *m/z* 1393.8 (A), 1626.2 (B) and 1951.7 (C) correspond to the $[M-7H]^{7-}$, $[M-6H]^{6-}$, and $[M-5H]^{5-}$ ions of unreacted dsDNA, respectively (where M is the mass of dsDNA). The spectrum in Fig. 3B shows groups of ions that correspond to dsDNA with 2, 3 or 4 molecules of CosD bound, but with varying numbers of sugars attached. Individual assignments for all ions are shown in Table 1.

In other studies, under similar conditions, we have found that 3 to 4 daunorubicin molecules are bound to 16 mer dsDNA, and as the daunorubicin concentration is increased (>9 : 1 DAU : dsDNA), a maximum of 5 molecules is bound[15,21]. Fig. 4 compares the abundances of DAU-dsDNA and CosD-dsDNA complexes in mixtures containing 0.5 : 1 to 9 : 1 drug : dsDNA as judged by negative ion ESI-MS. Relative abundances of species in the mixture were calculated by expressing the intensity of a particular species (*e.g.* dsDNA+3CosD) as a percentage of the sum of the intensities of all species in the mixture (including unreacted dsDNA, if any). The results from these experiments are shown in Fig. 4A (DAU) and 4B (CosD). When the dsDNA was treated with a 9-fold excess of CosD, the most abundant species in the mixture was that in which 4 molecules of CosSD were bound to dsDNA (M+4CosD). Under the same experimental conditions, but using DAU instead of CosD, the most abundant species in the mixture was that in which 4 molecules of DAU were bound to dsDNA. Therefore, under these experimental conditions, the binding behavior of DAU (Fig. 4A) was similar to that of CosD (Fig. 4B).

The relative abilities of CosD and DAU to bind DNA were investigated further in a competition experiment where the relative amounts of daunorubicin : CosD - dsDNA were 3 : 3 : 1. Results are shown in Fig. 5. Relative abundances of each species present in the mixture as judged by negative ion ESI-MS were calculated in the same way as the results in Fig. 4. In these experiments the two drugs bound equally well. The major ion corresponded to $[M+2CosD+2DAU-7H]^{7-}$ with $[M+2CosD+1DAU-7H]^{7-}$ at ≥90% of the abundance of the former. The most abundant ions were those from dsDNA molecules with 4 drug molecules bound (mixed DAU and CosD complexes).

Fig. 4. Relative abundances of complexes of DAU or CosD with dsDNA as judged by negative ion ESI-MS.



A. Relative abundances of DAU-dsDNA complexes in 0.5 : 1～9 : 1 ratios.

B. Relative abundances of CosD-dsDNA complexes in 0.5 : 1～9 : 1 ratios.

Relative abundances of species in the mixture were calculated by expressing the intensity of a particular species (*e.g.* dsDNA+3CosD) as a percentage of the sum of the intensities of all species in the mixture (including unreacted dsDNA, if any).

Table 1. Assignments of ions in negative ion ESI mass spectra shown in Fig. 4. Reaction of CosD with dsDNA.

Ions (Label; *m/z*)	Assignment
Fig. 3A	
A 1393.3	$[M-7H]^{7-}$
B 1626.2	$[M-6H]^{6-}$
C 1951.7	$[M-6H]^{6-}$
Fig. 3B	
A 1073.6	$[CosD\text{-}deoxyfucose\text{-}H]^{1-}$
B 1187.7	$[CosD\text{-}H]^{1-}$
C 1854.8	$[M+3(CosD\text{-}deoxyfucose)\text{-}7H]^{7-}$
D 1871.0	$[M+CosD+2(CosD\text{-}deoxyfucose)\text{-}7H]^{7-}$
E 1887.2	$[M+2CosD+(CosD\text{-}deoxyfucose)\text{-}7H]^{7-}$
F 1903.6	$[M+3CosD\text{-}7H]^{7-}$
G 2003.2	$[M+CosD+(CosD\text{-}deoxyfucose)\text{-}6H]^{6-}$
H 2022.4	$[M+2CosD\text{-}6H]^{6-}$
I 2024.5	$[M+CosD+3(CosD\text{-}deoxyfucose)\text{-}7H]^{7-}$
J 2040.6	$[M+2CosD+2(CosD\text{-}deoxyfucose)\text{-}7H]^{7-}$
K 2057.3	$[M+3CosD+(CosD\text{-}deoxyfucose)\text{-}7H]^{7-}$
L 2164.0	$[M+(3CosD\text{-}deoxyfucose)\text{-}6H]^{6-}$
M 2183.1	$[M+CosD+2(CosD\text{-}deoxyfucose)\text{-}6H]^{6-}$
N 2201.6	$[M+2CosD+(CosD\text{-}deoxyfucose)\text{-}6H]^{6-}$
O 2381.2	$[M+2CosD+2(CosD\text{-}deoxyfucose)\text{-}6H]^{6-}$
Fig. 3C	
A 1187.9	$[CosD\text{-}H]^{1-}$
B 1733.5	$[M+CosD\text{-}7H]^{7-}$
C 1813.9	$[M+4CosD\text{-}8H]^{8-}$
D 1903.3	$[M+3CosD\text{-}7H]^{7-}$
E 2073.1	$[M+4CosD\text{-}7H]^{7-}$
F 2220.7	$[M+3CosD\text{-}6H]^{6-}$
G 2418.8	$[M+4CosD\text{-}6H]^{6-}$

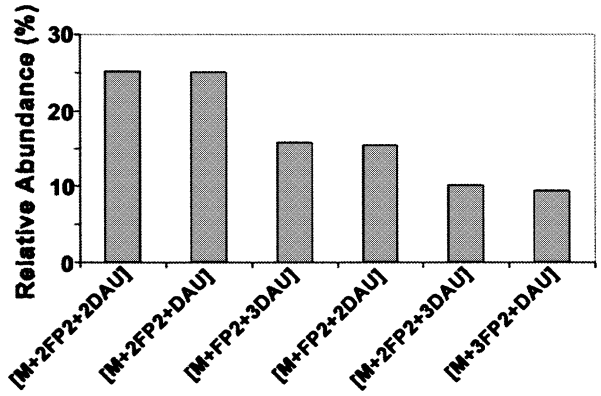
M = Mass of unreacted dsDNA

Discussion

Anthracycline antitumor antibiotics (doxorubicin and daunorubicin) have been used in chemotherapy for many years. Toxic effects on healthy cells are thought to involve generation of radicals through interaction of carbohydrate chains with iron. The oligosaccharide residues are also important in interactions with DNA, and in the ternary complex with topoisomerase II, since the sugars may interact with the minor groove while the planar ring system intercalates between base pairs. There have been several studies that have attempted to improve the pharmacological properties of anthracyclines by modification of the sugars[8,9,22]. In some cases, modification of the sugars affected the ability of the drug to poison topoisomerase II but did not affect the relative cytotoxicity of the anthracycline[9]. This raises the possibility that oligosaccharide moieties of anthracyclines might be modified to increase clinical efficacy, without increasing toxicity. The glycosylation pattern of CosD presents the possibility of new therapeutic properties for this anthracycline.

Gel DNA retardation is a very useful technique to detect intercalating molecules. CosD showed the grater retardation effect. Possible explanations for the better effect of CosD on the mobility of plasmid DNA compared with DOX or

Fig. 5. Relative abundances of anthracycline-dsDNA complexes in a 3 : 3 : 1 mixture of DAU : CosD : dsDNA.



Relative abundances of species in the mixture were calculated by expressing the intensity of a particular species (*e.g.* dsDNA+2CosD+2DAU) as a percentage of the sum of the intensities of all species in the mixture.

DAU include: (i) a larger number of CosD molecules are bound to the DNA than DOX, and/or (ii) the structure(s) of the CosD-dsDNA complexes causes greater lengthening of the DNA.

To our knowledge, there have been no investigations of the molecular interactions of an anthracycline containing a β-rhodomycinone aglycone chromophore *O*-linked to two trisaccharide chains with possible target molecules (*e.g.* DNA). ESI-MS is a gentle ionization technique that allows detection of large intact biomolecules such as proteins and DNA. The application of ESI-MS for studying structures of small molecules and for protein sequence analysis is well established[23], however, its use in screening non-covalent complexes is developing[24]. ESI-MS has been used to compare sequence preferences of various drugs for DNA[15,25]. There were no ions from complexes of dsDNA with CosD alone or DAU alone. These observations suggest that the binding constants for CosD and daunorubicin are similar in magnitude and are consistent with the proposal that these drugs bind at the same 4～5 sites on the 16 mer dsDNA.

The data show that ESI-MS has potential for use as a rapid sensitive method for screening interactions between anthracyclines and double-stranded DNA. These analytical results are surprising because DAU and CosD are quite dissimilar molecules, with large differences in the sugar composition. We are extending these studies to obtain a more detailed picture of the relative binding affinities of these anthracyclines for dsDNA. The binding of daunorubicin involves intercalation between DNA base pairs and it seems likely that CosD also binds by intercalation, but confirmation of this proposal will require more detailed structural analysis of these complexes.

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