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Mass spectrometric investigation of the DNA-binding properties of an anthracycline with two trisaccharide chains

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Mass Spectrometric Investigation of the DNA-Binding Properties of an Anthracycline with two Trisaccharide Chains

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Abstract

Cosmomycin D (CosD) is an anthracycline that has two trisaccharide chains linked to its ring system. Gel electrophoresis showed that CosD formed stable complexes with plasmid DNA under conditions where daunorubicin (Dn) and doxorubicin (Dx) dissociated to some extent during the experiments. The footprint and stability of CosD complexed with 10- and 16 mer DNA was investigated using several applications of electrospray ionization mass spectrometry (ESI-MS). ESI-MS binding profiles showed that fewer CosD molecules bound to the sequences than Dn or Dx. In agreement with this, ESI-MS analysis of nuclease digestion products of the complexes showed that CosD protected the DNA to a greater extent than Dn or Dx. In tandem MS experiments, all CosD-DNA complexes were more stable than Dn- and Dx-DNA complexes. These results support that CosD binds more tightly to DNA and exerts a larger footprint than Dn or Dx. ESI-MS investigations of the binding properties of CosD could be carried out rapidly and using only small amounts of sample.

Keywords: anthracyclines; non-covalent complex; electrospray ionization mass spectrometry; trisaccharide chains; gel mobility shift assay.

Introduction

Bioactive compounds produced by Gram-positive bacteria *Streptomyces*, a member of the actinomycetes order, have had widespread application in cancer chemotherapy. In particular, the anthracyclines daunorubicin (Dn, daunomycin) and doxorubicin (Dx, adriamycin) are very effective against a range of cancers (Figure 1). These compounds contain very similar aglycone moieties consisting of a tetracyclic ring system with adjacent quinone-hydroquinone groups with the amino sugar, daunosamine, attached to C-7 of ring A. The difference between these two compounds is that

the methyl group in the side chain of Dn is hydroxylated in Dx. This difference has a significant effect on the anti-cancer activities of these two compounds: Dn is used in chemotherapy against acute lymphoblastic or myeloblastic leukemias, while Dx is an essential element used in the treatment of breast cancer, childhood solid tumours, soft tissue sarcomas and aggressive lymphomas [1,2].

While these compounds have been used as an important part of chemotherapeutic regimens for more than 30 years, their mode(s) of action remain unclear [1]. The binding of anthracyclines to DNA (*in vitro*) has been extensively investigated using a range of biochemical and biophysical techniques, including equilibrium dialysis, fluorescence spectroscopy, observation of the melting temperature (T_m) of DNA and Dnase I footprinting analysis [3-7]. X-ray crystal structures of anthracyclines show that the planar ring systems of anthracyclines intercalate between base pairs of double-stranded (ds) DNA and that the sugar(s) lie in the minor groove [8,9]. Flow cytometry experiments have shown that anthracyclines accumulate in cell nuclei and bind to DNA [10]. The binding to nuclear DNA is expected to cause inhibition of replication and transcription supporting that the intercalation into DNA is an important mechanism in the activity against cancer cells. The structures of the anthracyclines allow for many interactions with other cellular components and macromolecules and other mechanisms for their anti-cancer activity include: (i) generation of free radicals that may damage DNA, lipids and proteins, (ii) alkylation of DNA, (iii) DNA cross-linking, (iv) interference with activities of enzymes that unwind DNA such as helicases, (v) direct membrane effects, (vi) DNA damage caused by inhibition of topoisomerase II, and (vii) induction of apoptosis in response to topoisomerase II inhibition [1,11]. The most widely accepted mode of action of the anthracyclines involves intercalation into DNA resulting in stabilization of a transient reaction intermediate of topoisomerase II activity in which DNA strands are cleaved and covalently linked to tyrosine residues of the enzyme [1].

The mechanisms outlined above are not exclusive of each other, and there are numerous overlapping cellular processes that can be affected. Given the complexity of the effects of anthracyclines in the cell, it is perhaps not surprising that there are no clear definitions of structural features that confer a specific biochemical or clinical effect. Further, this complexity contributes to difficulties in predicting the onset and severity of the negative aspects of anthracycline chemotherapy such as the development of resistance by tumour cells and cardiotoxicity [1]. Other than Dn and Dx, anthracyclines that have been used in the clinic include epirubicin (where the C-4′ hydroxyl of the daunosamine sugar in Dx is equatorial rather than axial), idarubicin (where the methoxy group at C-4 of the Dn aglycone is lacking), pirarubicin (which is a 4-tetrahydropyranol Dx) and aclacinomycin A (aclarubicin, where there are changes to the aglycone and there is a trisaccharide attached to C-7, see Figure 1). Idarubicin has a broader spectrum of activity than Dn that is thought to be related to increased cellular uptake (as a result of greater lipophilicity) and enhanced stabilization of the ternary drug-DNA-topoisomerase II complex [12,13]. Pirarubicin and aclacinomycin A show modest improvements against development of drug resistance compared with Dx and Dn [14].

There are numerous crystal structures of anthracycline-DNA complexes. The sugar residue is important in forming the stable ternary complex with topoisomerase II, and the sequence selectivity of anthracycline-stimulated DNA cleavage is influenced by the substituents at the C-3′ (primary amine in Dx and Dn) [13]. Furthermore, anthracyclines containing disaccharides such as the Dx derivative, MEN10755, have been shown to have activity against different cancers, and this compound is active against Dx-resistant tumours [15,9]. In order to produce anthracyclines with different pharmacological profiles, clinical efficacy and diminished side effects, a range of strategies has been employed to alter the structures of aglycones and/or the sugar residues. These include screening natural products, chemical synthesis of novel aglycones [16], and genetic manipulation of anthracycline-producing bacteria [2,17].

Previously we reported the purification and characterisation of an anthracycline from *Streptomyces olindensis* ICB20 containing a β-rhodomycinone aglycone *O*-linked at each of C-7 and C-10 to trisaccharide chains comprising L-rhodosamine, 2-deoxy-L-fucose and L-rhodinose [18]. This compound was identified as cosmomycin D (CosD) which was originally isolated from *Streptomyces cosmosus* in the mid-1980s [19,20]. In that early work, CosD was shown to induce differentiation in cultured Friend F5-5 leukemia (mouse) cells under conditions where doxorubicin, daunorubicin and aclacinomycin A had no measureable effect. This implies that the structural features of CosD favour a different set of intermolecular interactions in the cell. Since these initial observations there had been no further investigations of the properties of this compound. Our recent work reported the first investigations of the DNA-binding properties of CosD [18]. A preliminary survey of the binding of CosD to double-stranded (ds) 16 mer DNA using electrospray ionization mass spectrometry (ESI-MS) showed that 3-4 CosD molecules bound to the DNA compared with 4-5 for Dn. A gel mobility shift assay (using only one concentration of drug) showed that CosD decreased the mobility of plasmid DNA to a greater extent than Dx or Dn [18].

The current work is the first extensive study of the DNA-binding properties of CosD, an unusual anthracycline containing two trisaccharide chains. There have been few anthracyclines with two trisaccharide chains that have been identified and very few detailed studies of their DNA-binding properties. Ditrisarubicin (Dtr) contains two trisaccharides, but the final two sugars of each chain are fused (Figure 1). Anthracyclines that have different structural features are likely to exhibit different interactions with cellular biomolecules and may have anticancer activities that are different to those of anthracyclines currently used in the clinic. The DNA-binding properties of CosD compared with Dx and Dn are reported in the current work, and the merits of ESI-MS as a first pass for screening compounds that interact with DNA are briefly discussed. ESI-MS was used to: (i) compare the number of drug molecules required to saturate the binding sites of a 16 mer dsDNA sequence, (ii) detect and compare DNA fragments resulting from enzymatic digestion

(phosphodiesterase I and Dnase I), and (iii) investigate the stability of drug-DNA complexes in tandem mass spectrometry experiments. The effect of increasing concentrations of Dx, Dn and CosD on unwinding supercoiled plasmid DNA was investigated using agarose gels.

Materials and methods

All reagents were of the highest grade commercially available and MilliQ™ water was used in all experiments.

Oligonucleotides

Oligonucleotides (Figure 2) were purchased from Geneworks (South Australia) as the trityl-off derivatives and were dissolved in 10 mM ammonium acetate for purification using a Waters HPLC. Samples were loaded onto a C-18 octadecylsilyl column (8 x 100 mm, Waters Delta Pak Radial Pak Cartridge) equilibrated at 1 mL min^{-1} with 10 mM ammonium acetate and were eluted from the column using an acetonitrile gradient (0-60% over 30 min). The oligonucleotides free of sodium were freeze-dried using a Savant Speed-Vac and redissolved in 100 mM ammonium acetate, pH 7.5. The concentration of ssDNA was determined by measuring the absorbance at 260 nm and applying the molar extinction coefficients for the individual bases of 12010, 15200, 7050 and 8400 $M^{-1}cm^{-1}$ for guanine, adenine, cytosine and thymine, respectively. Double-stranded DNA (to give 1 mM) was obtained by annealing the appropriate relative amounts of the complementary single strands in 100 mM ammonium acetate, pH 7.5, by heating to 90 °C for 15 min and allowing to cool slowly to room temperature.

Anthracyclines

Daunorubicin and doxorubicin were purchased from Sigma-Aldrich (St Louis, USA) and were used without further purification. Cosmomycin D from *Streptomyces olindensis* ICB20 was purified from methanol extracts of liquid cultures as previously described [18]. In some experiments, pure CosD was obtained from relatively crude culture extracts by dissolving the dried

methanol extract in water, sonicating for 5 min and centrifuging in an Eppendorf microfuge (10,000 rpm, 4 °C, 15 min). The supernatant was frozen for 24 hours, thawed slowly to room temperature, and centrifuged as before. The freeze-dried supernatant was dissolved in water (50 mg/mL) and filtered using a 0.45 μm nylon syringe filter. The filtrate was purified by HPLC using a Jupiter C4 column (Phenomenex, 10 x 250 mm, 5 μm, 300 Å) equilibrated at 3 mL/min with 100 mM ammonium acetate, pH 7.5, and eluted using a 0-65% acetonitrile gradient over 65 min. Cosmomycin D eluted at 35.2 min and was obtained as a red-purple powder after freeze-drying. All anthracycline stock solutions (1 mM for Dn and Dx and 0.5 mM for CosD) were prepared in 100 mM ammonium acetate, pH 7.5.

Preparation of anthracycline-DNA complexes

Equimolar (1:1) anthracycline-DNA mixtures contained 78 μL of 100 mM ammonium acetate, pH 7.5, 1 μL of the appropriate dsDNA solution (1 mM) and 1 μL of stock anthracycline (77 μL of ammonium acetate, 1 μL of dsDNA and 2 μL of CosD). Other anthracycline-DNA mixtures (1:2, 1:3, 1:4, 1:5, 1:7, 1:9, 1:11, 1;13) were prepared by altering the relative amounts of anthracycline and ammonium acetate, but maintaining the total volume of 80 μL. After an equilibration time of 10 minutes, the solution was diluted just prior to ESI-MS analysis by adding 120 μL of 100 mM ammonium acetate, pH 7.5. This gave a final dsDNA concentration of 5 μM.

Nuclease digestion

Anthracycline-DNA complexes (1:6, 1:6, 1:2.5 for dsDNA:Dn, dsDNA:Dx and dsDNA:CosD, respectively) were prepared as described above. Phosphodiesterase I (from *Crotalus adamanteus* venom) and DNase I (type II, from Bovine pancreas) were obtained from Sigma-Aldrich (St Louis, USA). The enzymes were diluted into water, to give absorbances at 280 nm of 0.158 and 0.870, respectively (using 0.1 cm pathlength quartz cuvettes). Nuclease digestion was commenced by addition of 10 μL of PdI or 20 μL for DNase) to 50 μL of anthracycline-DNA

complex (25 °C). At each time point, 10 μL of the reaction mixtures were added to 90 μL of 5 mM ammonium acetate, 50% in acetonitrile to stop the reaction and immediately analysed by ESI-MS (see below). The products of nuclease digestion were analysed by reference to Mongo Oligo Mass Calculator (http://library.med.utah.edu/masspec/mongo.htm).

Agarose gel electrophoresis

The plasmid DNA (pUC9) was grown and purified using a QIAGEN plasmid Maxi Kit, and its concentration was calculated from its absorption at 260 nm. The DNA (pUC9, 50 μg/ml) and drug (1 to 20 μM for Dn and CosD and 1 to 50 μM for Dx,) stock solutions were prepared in 50 mM ammonium acetate, pH 7.5. The electrophoresis buffer was made by dilution (1:10) of the stock buffer solution (EDTA 10 mM, Boric acid 445 mM, Tris Base, Ultrol grade 445 mM, in 1 L of water). For a typical sample, 5 μL of stock anthracycline solution (different concentrations, see Results and Discussion) were added to 5 μL of plasmid DNA (50 μg/ml). The 5 μL of anthracycline solution was replaced by 5 μL of 1:10 buffer in the control samples and by 5 μL of HindIII for opening of the supercoiled DNA. The solutions were equilibrated at room temperature for 15 min before adding 2.5 μL of dye (0.25% bromophenol blue in sucrose 40 % *w/v*), and 10 μL of these mixtures was loaded onto a 1% agarose gel. The gels were electrophoresed for 5 h at 30 V, stained (15 μL of 10 mg/mL of ethidium bromide in 500 mL of water) for 35 min and then rinsed in water for 20 min. Gels were photographed using a Bio-Rad Molecular Imager Gel Doc XR System.

Mass spectrometry

Samples were analysed using a Waters Q-ToF Ultima ESI mass spectrometer equipped with a Z-spray ionization source and a quadrupole mass analyzer with a *m/z* range of 32,000 (not necessary to have this *m/z* range for these experiments). Samples were injected to the mass spectrometer using a Harvard Model 22 syringe pump (Natick, MA, USA). All spectra were acquired using a MCP

potential of 2100 V, RF lens energy of 70 V, and the cone and capillary voltages were 100 V and 2.5 kV, respectively. The source temperature was 80 °C and the desolvation gas flow was 300 L/hr at a temperature of 100 °C. Spectra were acquired in negative ion mode over the range *m/z* 500-4000 and typically 10 scans were summed to obtained a representative spectrum. In the nuclease digestion experiments, the cone voltage was set to 250 V, the source temperature to 60 °C, the desolvation gas temperature to 200 °C and typically 40 scans were summed to obtain a representative spectrum; all the other parameters were the same as above. The instrument was calibrated using a CsI solution (1 mg/mL) over the same *m/z* range. Tandem mass spectrometry experiments (MS/MS) were carried out under the same conditions with argon as the collision gas set to a pressure of 15 psi. The collision energy was varied form 0 to 28 V to establish a relationship between the amount of energy applied and the dissociation products observed. All experiments were repeated at least twice and the results were reproducible.

Results and Discussion

Agarose gel electrophoresis of anthracycline complexes with pUC9

Intercalators stack between base pairs of dsDNA, causing it to lengthen, unwinding the negative supercoil, and decreasing the mobility through agarose gels [21]. The migration of complexes of Dx, Dn and CosD with the plasmid pUC9 through agarose gels was investigated to compare the degree of unwinding induced by intercalation. Plasmid DNA was treated with a range of concentrations of the different anthracyclines and the mixtures were run on gels and stained using ethidium bromide. Figure 3 shows the gels for Dn (A), Dx (B) and CosD (C). In each of the gels, lanes 1 and 15 contained only pUC9. The two major bands are from negatively supercoiled closed circular DNA (sc) and open circles (oc). Lanes 2 and 16 contained pUC9 linearized by treatment with HindIII. Lanes 4 to 13 contained pUC 9 with increasing anthracycline concentrations. The

drug-to-nucleotide ratios, r_b, for Dn (Figure 3A) and CosD (Figure 3C), were the same in corresponding lanes. In the case of Dx, (Figure 3B), a greater range of concentrations was used since higher r_b values were required to cause effects similar in magnitude to those observed for Dn. With respect to the drug-to-nucleotide ratio, lane 4 for Dx (r_b, 0.0130) is comparable with lanes 6 for Dn and CosD (r_b, 0.0136) and lane 7 for Dx (r_b, 0.0522) is comparable with lanes 13 for Dn and CosD (r_b, 0.0515).

Comparison of the mobilities of plasmid DNA complexes with Dn (Figure 3A), Dx (Figure 3B) or CosD (Figure 3C) reveals different properties. As the concentration of Dn increased, the mobility of supercoiled (sc) DNA decreased, consistent with results observed for other intercalators [22]. When r_b reached 0.0461 (lane 12), most of the DNA was in the relaxed form (oc). Some smearing was evident, particularly in lanes 12 and 13, suggesting that some of the Dn dissociated from the DNA during electrophoresis. Similar behavior was observed for Dx, although higher concentrations of the drug were required to observe a similar shift in mobility. The greatest mobility shift was observed for CosD. Furthermore, the smearing that was observed for Dn and Dx was not present. In contrast, resolvable bands were present, representing stable CosD-plasmid complexes containing different numbers of bound CosD and having different superhelical densities. Under the experimental conditions, a saturation point was reached for Dn and CosD where no further changes were discernible under the experimental conditions as the anthracycline concentration was increased (lane 12, r_b 0.0461 for Dn and lane 10, r_b 0.0353 for CosD). This corresponds to a molecule of Dn for every ~22 nucleotides and a molecule of CosD for every 28 nucleotides of pUC9 DNA.

At high concentrations of the anthracyclines, the overall intensity of the bands in a given lane was diminished compared with the overall intensity at lower concentrations, possibly because the binding of the anthracycline hindered binding of ethidium bromide that was used for fluorescent detection of the DNA. This was supported by experiments where complexes of Dn, Dx or CosD

with 16 mer dsDNA (Rt1, see below) were prepared and analysed using 4% agarose gels. When the gels were stained with ethidium bromide, bands from DNA were clearly evident in lanes loaded with Dn- or Dx-DNA mixtures. In contrast, bands in lanes loaded with CosD-DNA mixtures were only weakly stained and the mobility of the band was decreased relative to the Dn- or Dx-treated DNA (not shown). The inability to stain the 16 mer dsDNA suggests that the ethidium bromide could not displace the CosD from the DNA, consistent with the proposal that CosD binds more tightly to DNA than Dn or Dx.

Under some experimental conditions, as the concentration of an intercalator is increased, closed circular DNA unwinds to the point that it comigrates with open circular DNA. At higher concentrations again, the DNA becomes positively supercoiled and the mobility increases [23]. This behavior was not observed under our experimental conditions; mobility of the open circular DNA (oc) also decreased as the concentration of anthracycline increased, especially in the case of CosD. This is consistent with the positive charges on CosD (two tertiary amines) neutralizing negative charges on DNA as has been observed for other positively charged DNA-binding compounds [22]. The two effects: DNA-unwinding and the effect of positive charge complicates calculation of an unwinding angle as a result of intercalation of CosD.

ESI-MS of anthracycline-DNA complexes

There are now many examples from our laboratory and others where non-covalent drug-DNA complexes have been detected and analysed by ESI-MS [24-29]. These experiments are routine in our laboratory and others, and preliminary screening of drug-binding to previously prepared 16 mer DNA sequences can be carried out in approximately two hours. Figure 2 shows the DNA sequences used in the current work, along with the masses of the dsDNA. These were 10 mer (F0AT) and 16 mer (Rt1) dsDNA sequences, as well as F0AT with various single- or double-stranded poly dA extensions. In the "screening" experiments, Rt1 was mixed with various concentrations of Dn, Dx

or CosD (dsDNA: anthracycline of 1:1, 1:3, 1:5, 1:7, 1:9, 1:11 and 1:13 for Dn and Dx, 1:1, 1:2, 1:3, 1:4 and 1:5 for CosD), and the mixtures were analysed by ESI-MS. As the concentration of anthracycline was increased, the spectra showed that increasing numbers of anthracycline molecules bound to the DNA. Figure 4 shows negative ion ESI mass spectra of Rt1 alone and of mixtures with each anthracycline where the DNA was saturated (*i.e.* when the anthracycline concentration was increased further, no new complexes appeared in the ESI mass spectra). It should be noted that electrospray ionization generates ions carrying different numbers of charges. For free dsDNA and for all the anthracycline complexes with dsDNA, the most abundant ions were those carrying six negative charges. In the spectrum of Rt1 alone (Figure 4A), the $[Rt1\text{-}6H]^{6-}$ ion was at mass/charge (*m/z*) 1626.8, in good agreement with the calculated value of 1626.2 (mass, 9763.5 Da). Ions corresponding to $[Rt1\text{-}5H]^{5-}$ (*m/z* 1951.8), $[Rt1\text{-}7H]^{7-}$ (*m/z* 1393.8) and single-stranded DNA (*m/z* 1201.0 and 1237.7), were also present but in low-moderate abundance. Rt1 was saturated with Dn when the dsDNA: anthracycline ratio was 1:11 (Figure 4B). The most abundant ion was from Rt1 + 4 Dn (*m/z* 1978.6, mass 11873.6 Da) with other ions from complexes containing two to six Dn molecules. Rt1 was saturated with Dx and Cos D when the ratios were 1:13 and 1:4, respectively, and the spectra of these mixtures are shown in Figures 4C and D.

The relative abundances of each complex in these mixtures as judged by ESI-MS (saturating amounts of anthracycline) were compared by summing the intensities of ions from each complex and expressing this as a percentage of the sum of the intensities of all ions in the spectra. A graph of these data (Figure 4E) gives the anthracycline binding profile for the DNA sequence and is a convenient way to compare binding data at a glance. The most abundant complex in the Dn- and Dx-dsDNA mixtures contained four anthracycline molecules (*e.g.* Rt1 + 4Dn), while for CosD the most abundant complex contained three CosD molecules (Rt1 + 3CosD). The results for Dn are in agreement with previous ESI-MS results analyzing binding of anthracyclines to 16 mer dsDNA [25,26] and our preliminary ESI-MS analysis of CosD [18]. The data show that the dsDNA can

bind fewer molecules of CosD compared with Dn and Dx, consistent with a larger "footprint" for CosD on the 16 mer dsDNA.

While the rings of anthracyclines intercalate between DNA base pairs, the amino sugars bind in the minor groove of DNA [30]. There are no X-ray or NMR structures available for a CosD-DNA complex or for any anthracycline containing two trisaccharide chains. A structure of aclacinomycin bound to 6 mer dsDNA shows that its single trisaccharide chain also lies in the minor groove of the DNA [31]. It seems likely that the trisaccharide chains of CosD will also bind in the minor groove. ESI-MS experiments cannot directly comment on the structure of the DNA complexes detected. It is possible that some of the less abundant complexes may have been bound non-specifically, perhaps as a result of an electrostatic interaction between the positively charged drugs and the phosphodiester backbone of DNA. This possibility was minimized in these experiments by using a high salt concentration (100 mM) and low concentrations (μM) of analyte [32].

In order to test that the CosD-DNA complexes were "specific", that is, resulted from intercalation between base pairs of dsDNA, the 10 mer sequence, F0AT, was modified by addition of single-stranded (ss) tails (poly dA of either 10 or 20 nucleotides in length; see Figure 2 for sequences). If double-stranded DNA is required for binding, and non-specific electrostatic-only interactions are minimal, then the maximum number of anthracycline molecules that bind to the DNA (binding profile) is expected to be unchanged when ssDNA tails are added to F0AT. Figure 5 shows the relative abundances of different anthracycline-DNA complexes (binding profiles) in different saturating mixtures of Dn, Dx or CosD with F0AT (Figure 5A), F10A1 (Figure 5B; a poly dA_{10} tail was added to one of the strands of F0AT), F20A2 (Figure 5C; a poly dA_{20} tail was added to one of the strands of F0AT), and F20A1A2 (Figure 5D; poly dA_{20} tails were added to both strands of F0AT, giving a forked DNA structure). The predominant complex in the mixture of the 10 mer dsDNA with Dn (Figure 5A), was F0AT + 3Dn in the saturating 1:13 mixture and F0AT +

2CosD predominant in the saturating mixture with CosD (1:2.5). Since the F0AT sequence is six base pairs shorter than Rt1 (Figure 2), it is expected that fewer anthracycline molecules will be able to bind as a result of steric hindrance. The results for Dn (3 bound as the major complex for F0AT compared with 4 for Rt1) and CosD (2 for F0AT compared with 3 for Rt1) are consistent with this. The major feature of these binding profiles is that they were similar for all the sequences: the double-stranded F0AT, F0AT with single-strand extensions and the forked structure, supporting that the anthracyclines bound to the double-stranded region through intercalation.

Similar observations were made for Dx, but fewer Dx molecules bound to all the sequences containing F0AT than for Dn. This was not evident in the studies using the 16 mer sequence Rt1 where the binding profiles of Dn and Dx substantially overlapped (Figure 4E). This observation is consistent with a sequence specificity of Dx that is not exhibited by Dn. X-ray structures of Dn and Dx complexes with dsDNA have previously revealed structural differences that might account for the different clinical activities of these two drugs [8].

Nuclease footprinting analysed by ESI-MS

Footprinting studies using nuclease and/or chemical digestion have been used to gain insight into the binding sites of anthracyclines on DNA [7,33]. Typically, a plasmid fragment of several hundred base pairs is isolated, labelled, and mixed with the anthracycline prior to addition of Dnase I. The fragments produced are isolated by precipitation and analysed using denaturing polyacrylamide gels. In this way, it has previously been demonstrated that anthracyclines with a greater number of sugar residues have a greater footprint on DNA [7]. In the current work, a simple, rapid method was used to screen the footprint of Dn, Dx and CosD on the 16 mer Rt1 DNA. DsDNA-anthracycline mixtures were chosen that would give as the major complexes [Rt1 + 2 anthracyclines] and [Rt1 + 3 anthracyclines] as judged by ESI-MS binding profiles. The dsDNA: anthracycline mixtures were 1:6, 1:6 and 1:2.5 for Dn, Dx and CosD respectively. This was followed by treatment with either phosphodiesterase I (PdI, an exonuclease) or Dnase I (an

endonuclease) for various lengths of time. The products from the digestions were analysed by ESI-MS after dilution of the mixtures into a low ionic strength solution containing acetonitrile. When dsDNA is diluted into this solution and analysed by ESI-MS, the DNA is denatured to single strands. Figure 6A-D show the mass spectra that were obtained after Rt1 (A) or its complexes with Dn, Dx or CosD (B-D, respectively), were treated with PdI for 30 minutes. When Rt1 was treated with PdI, most of the ions in the mass spectrum were from nucleotides arising from enzymatic digestion of the DNA, with little intact DNA remaining. Some of the major nucleotide products were from the single strand shown in the 3′→5′ direction in Figure 2, Rt1B $[G1:G14]^{4-}$, Rt1B $[G1:G14]^{3-}$ and $[dsRt1 - GA]^{5-}$ at *m/z* 1077.1, 1436.4 and 1823.1, respectively.

When the anthracycline-DNA complexes were treated with PdI for 30 min, there were more ions (and of greater intensity) from intact DNA, showing that anthracycline binding protected the DNA from exonuclease cleavage. The amounts of intact DNA were similar for Dn (B) and Dx (C), while the spectrum for the CosD-Rt1 mixture (D) showed a substantially greater number (and summed intensity) of ions. These data are represented graphically in Figure 6E where the intensities of ions from intact DNA were summed at each time point and expressed as a percentage of the intensities of all ions in a spectrum of Rt1 DNA treated in the same way, but without PdI. The results for doxorubicin are not shown because they were very similar to those for daunorubicin. This is consistent with the earlier ESI-MS data (Figure 4) that showed that the binding profiles of Dx and Dn for Rt1 were similar, implying that the footprint on the DNA was similar. The data for digestion with Dnase I are shown in Figure 6F. Similar trends were observed for both enzymes. The experiments were carried out on several occasions and the results were reproducible. The amount of intact DNA remaining was greater when CosD was bound to Rt1, again consistent with a larger footprint on the DNA. The only deviation from this was when the mixtures had been treated with PdI for 1 minute where the extent of digestion of Rt1-CosD was approximately the same as that for free Rt1, and lower than for Rt1-Dn. Since PdI is a 3′ exonuclease, this may reflect the location of

binding of Dn and CosD on Rt1. That is, CosD molecules might not be bound as close to the 3′ end of the DNA as Dn allowing PdI greater access to unprotected DNA. As digestion proceeds, however, the more extensive binding of CosD compared to Dn (or Dx) impeded the phosphodiesterase.

There has been one other DNA footprinting study of an anthracycline with two trisaccharide chains. This was ditrisarubicin, in which the terminal two sugars are fused (Figure 1). In these experiments, the DNA was a 375 base pair fragment of pBR322 DNA [7]. This work also showed that ditrisarubicin protected a greater number of DNA base pairs from chemical and Dnase I cleavage than the less extensively glycosylated daunorubicin.

After annealing the commercially available DNA strands, an entire nuclease digestion experiment analysed by ESI-MS could be completed in an hour and using only 2 picograms of CosD. The experimental procedure could be modified in several ways to provide further information, and we are currently carrying out experiments to standardize these methods. For example, analysis of the digestion products at early time points or using low nuclease concentrations will provide information about where the drug molecules are bound on the sequence.

Stability of anthracycline-DNA complexes judged by tandem mass spectrometry

In tandem mass spectrometry experiments (ESI-MS/MS), an ion is selected (in the quadrupole in the case of a quadrupole time-of-flight, Q-ToF, instrument) and accelerated through a collision cell containing a low pressure of inert gas such as argon. Complexes dissociate (or covalent bonds are broken) when the kinetic energy is converted to internal energy as a result of collisions with the argon. The products of the dissociated complex are then analyzed in the ToF mass analyzer. The more stable the complex, the greater the collision energy that needs to be applied to dissociate the complex for a given dissociation pathway. The stabilities of Dn- and Dx-dsDNA complexes have previously been compared in this way [28,29].

In the current work the ESI-MS/MS dissociation behaviors of Dn- and Dx-Rt1 complexes were compared with results previously reported in the literature [28,29] for Dn- and Dx-DNA complexes, and with results obtained here, for the first time, for the dissociation of CosD-Rt1 complexes. In contrast with other methods (*e.g*. circular dichroism spectroscopy), ESI-MS reveals a mixture of complexes (Figure 4B-D) where one or more anthracycline molecules are bound to the DNA. This provides an opportunity to test the stabilities of each of these complexes. Furthermore, because the electrospray ionization process generates ions with different numbers of charges, dissociation pathways for complexes carrying different numbers of charges can be compared. Since the ions carrying six and five negative charges were the most abundant in our mass spectra, these were chosen for analysis over a range of collision energies. For the ions $[Rt1 + 1,2 \text{ or } 3\ Dn]^{6-}$, $[Rt1 + 1,2 \text{ or } 3\ Dx]^{6-}$, $[Rt1 + 1,2 \text{ or } 3\ Dn]^{5-}$ and $[Rt1 + 1,2 \text{ or } 3\ Dx]^{5-}$ the major dissociation pathway was loss of a neutral drug molecule, followed by (only in the case of the 6- ions) dissociation of the dsDNA into single-strands. In each spectrum, there were also ions corresponding to the different complexes with DNA where one guanine base had been lost. These observations are in agreement with other studies using different DNA sequences (12- and 14 mer sequences also with terminal guanine residues) [28,29].

For a given collision energy and for all complexes, the greater stability of the CosD-Rt1 complexes was evident in that a higher energy was required to dissociate CosD from the DNA. To illustrate this, Figure 7 shows negative ion ESI-MS/MS spectra (collision energy, 10 V), of the ions carrying 5 negative charges of Rt1, Rt1 + 2 Dn, Rt1 + 2 Dx and Rt1 + 2CosD. The precursor ion (the ion selected for dissociation in the collision cell) is designated by an arrow in each spectrum. Under these conditions, Rt1 was not separated to single strands, but an ion of low abundance corresponding to the loss of a guanine from Rt1 was evident (Figure 7A). For Rt1 + 2Dn (B) and Rt1 + 2Dx (C), the products were Rt1, Rt1 +1Anthracycline and the corresponding complexes that had lost guanine. The complex with Dx was somewhat more stable than that with Dn as evidenced

by the greater abundance of the product ions in the ESI-MS/MS spectrum for Rt1 + 1Dn (Figure 7B) compared with Rt1 + 1Dx (Figure 7C), in agreement with other work [29]. This observation has been explained in terms of an increase in activation enthalpy for DNA complexes with Dx compared with Dn as a consequence of the extra H-bond that may be present in complexes with Dx [29,6]. At this collision energy, and at all collision energies tested (up to 24 V), no CosD molecules dissociated from the DNA. The results were the same for Rt1 complexes with 3 molecules of each of the anthracyclines. At high collision energies (26 V), it was possible to dissociate all of the Dn and Dx from the DNA.

The complexes Rt1 + xCosD carrying six negative charges were also more stable than the corresponding ions for complexes with Dn and Dx. In contrast to the complexes carrying five negative charges, it was possible to dissociate one CosD from Rt1 + 3CosD giving Rt1 + 2CosD carrying only five charges. This tightly bound complex resisted further loss of CosD molecules as the collision energy was increased to 24 V; it was not possible to apply higher collision energies and maintain the quality of the mass spectra. Similar behavior has been observed for the dissociation of actinomycin D from 12 mer dsDNA using a Q-ToF mass spectrometer similar to the one used in the current experiments. This intercalator contains two bulky cyclic peptides that lie in the minor groove [29]. A complete and detailed study of the dissociation of CosD-DNA complexes carrying different numbers of charges, ion mobility experiments to examine the distribution of conformations of the complexes, and a discussion of the possible explanations for the differences in stabilities is to be reported elsewhere.

Conclusions

This is the first extensive study of the DNA-binding properties of CosD, an unusual anthracycline with two trisaccharide chains. Structures of less extensively glycosylated anthracyclines with DNA show that the sugars bind in the minor groove of DNA. It is likely that the sugars of CosD also bind

in the minor groove and that the increased number of non-covalent interactions result in a complex that has greater stability and a larger footprint on DNA. The greater stability of CosD complexes compared with that of the clinically used daunorubicin and doxorubicin was suggested by gel mobility shift assays in which stable CosD-pUC9 complexes differing by one superhelical turn were evident. Various experiments employing ESI-MS as a technique were used to compare the differences in DNA-binding properties. Since CosD is a purified natural product, it is available only in milligram quantities. The sensitivity and the speed of analysis of the ESI-MS technique enabled these comparisons to be carried out very efficiently, using less than a milligram of CosD in all experiments once the methodology was developed. Binding profiles where ESI-MS was used to analyse the stoichiometries of the anthracycline-Rt1 DNA complexes showed that the DNA was saturated with CosD at lower concentrations and that a smaller number of molecules could bind than for Dn and Dx. Nuclease footprinting experiments where the products of digestion were analysed by ESI-MS showed that CosD protected the DNA to a greater extent than Dn and Dx, again consistent with the sugars of CosD covering a larger number of DNA base pairs. Tandem mass spectrometry experiments showed that all CosD complexes were more stable to dissociation than their Dn and Dx counterparts.

The glycosylation pattern of CosD affords it different chemical properties and opportunities for a different array of molecular interactions from Dn and Dx. This may affect cellular uptake and its intracellular behavior, making it of interest as a compound that may have different therapeutic properties to drugs currently used in the clinic. The interaction of anthracyclines with topoisomerase II has been proposed as a mechanism for their anticancer activities [1]. The sugar residues of anthracyclines with DNA are important in the interactions of these drugs with topoisomerase II. For example, an axial orientation of the second sugar relative to the first in disaccharide analogs of idarubicin has been shown to be required for topoisomerase poisoning activity and cytotoxicity [34]. In view of this, the different glycosylation pattern of CosD compared

with drugs currently used in the clinic might afford it a different anticancer profile. Another potential application of Cos D is suggested by early studies of the effects of CosD on mammalian cells showing that it stimulated cell differentiation under conditions where Dn and Dx had little effect [20].These effects on cell differentiation [20] might make CosD useful as a reagent for inducing this behavior in cell biology experiments or for probing the underlying mechanisms involved in cell differentiation.

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.

FIGURE LEGENDS

Figure 1: Structures of some anthracyclines: daunorubicin (Dn, daunomycin), doxorubicin (Dx, adriamycin), cosmomycin D (CosD), aclacinomycin (Acl) and ditrisarubicin (Dtr). The numbering system for the aglycone ring system is shown at the top of the Figure.

Figure 2: DNA sequences used in this work. The mass of each dsDNA is shown on the right hand side.

Figure 3: Agarose gel electrophoresis of anthracycline-pUC9 complexes. A. Dn-pUC9, B. Dx-pUC9, C. CosD-pUC9. In each gel lanes 1 and 15 contain plasmid only; lanes 2 and 16 contain pUC9 linearized by treatment with HindIII. No samples were loaded in lanes 3 and 14. The drug-to nucleotide ratios, r_b, for gels A and C, lanes 4 to 13, were 0.0027, 0.0081,0.0136, 0.0190, 0.0244, 0.0298, 0.0353, 0.0407, 0.0461 and 0.0515, respectively For gel B, the ratios for lanes 4 to 13 were 0.0130, 0.0261, 0.0391, 0.0522, 0.0652, 0.0782, 0.0913, 0.1043, 0.1173 and 0.1304, respectively. The bands corresponding to supercoiled closed circular DNA (sc) and open circles (oc) are marked by arrows.

Figure 4: Negative ion ESI mass spectra of saturating mixtures of anthracyclines with Rt1 DNA. A. Rt1 alone; B. Rt1: Dn, 1:11; C. Rt1: Dx, 1:13; D. Rt1: CosD, 1:4. ○ ss- or dsDNA alone; □ Rt1 + 2 anthracyclines; △ Rt1 + 3 anthracyclines; ● Rt1 + 4 anthracyclines; ▲ Rt1 + 5 anthracyclines; ■ Rt1 + 6 anthracyclines; ✚ Rt1 + 7 anthracyclines. E. The intensities of ion from each complex in a spectrum were summed and expressed as a percentage of the sum of the intensities of all ions in the spectrum to give binding profiles of each anthracycline to Rt1. ▬ Rt1:Dn , 1:11; ▪▪▪ Rt1:Dx, 1:13; + ▬ Rt1:CosD , 1:4.

Figure 5: Binding profiles of the anthrayclines for the 10 mer dsDNA, F0AT and poly dA extensions (see Figure 3 for sequences). A. F0AT; B. F10A1; C. F20A2; F20A1A2. Data are shown for 1:11, 1:13 and 1:2.5 mixtures of the DNA with Dn (▬▬), Dx (▪▪▪) and CosD (▬), respectively.

Figure 6: Analysis of nuclease digestion experiments using ESI-MS. A-D. ESI mass spectra of phosphodiesterase I digestions (30 min) after dilution into 5 mM ammonium acetate, 50% in acetonitrile. A. Rt1 alone; B. Rt1 + Dn; C. Rt1 + Dx; D. Rt1 + CosD. ○ Digested DNA fragments; ● Intact free DNA (ss- or ds-); ■ Intact ssDNA + anthracycline(s); ▲ Intact dsDNA + anthracycline(s). Comparison of amount of intact DNA remaining in nuclease digestion mixtures is shown in E. Phosphodiesterase I; F. Dnase I. The bottom (hashed) section of the bars represent intact free ss- or dsDNA, the middle pale gray section of the bars represent the relative amount of intact ssDNA + anthracycline(s), and the small top section (darker gray) represents intact dsDNA + anthracycline(s).

Figure 7: Negative ion tandem mass spectra (ESI-MS/MS) of the 5- charge states of: A. Rt1 (*m/z* 1951.9); B. Rt1 + 2 Dn (*m/z* 2163.1); C. Rt 1 + 2 Dx (*m/z* 2169.7); D. Rt1 + 2 CosD (*m/z* 2428.0). ● Rt1; ○ Rt1 – 1G; ▲ Rt1 + 1 anthracycline; △ Rt1 + 1 anthracycline – 1G; ■ Rt1 + 2 anthracyclines; □ Rt1 + 2 anthracyclines – 1 G. The precursor ion in each spectrum is indicated by an arrow.

Figure 1

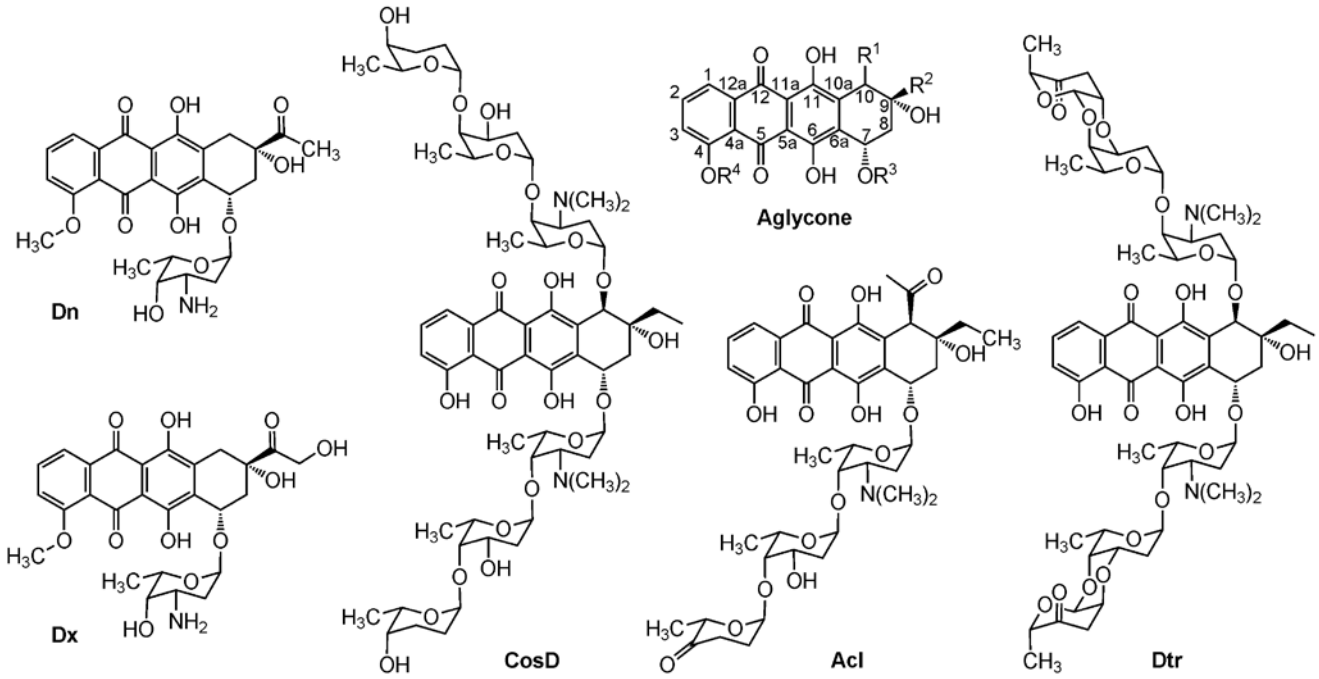

Figure 2

Rt1	5’ - CTCGTCCGATTCGATC - 3’ 3’ - GAGCAGGCTAAGCTAG - 5’	9763.5 Da
F0AT	5’ - TGCTCGGACG - 3’ 3’ - ACGAGCCTGC - 5’	6507.0 Da
F10A1	5’ - TGCTCGGACGAAAAAAAAAA - 3’ 3’ - ACGAGCCTGC - 5’	9189.1 Da
F20A2	5’ - TGCTCGGACG - 3’ 3’ - ACGAGCCTGCAAAAAAAAAAAAAAAAAAAA - 5’	12321.2 Da
F20A1A2	5’ - TGCTCGGACGAAAAAAAAAAAAAAAAAAAA - 3’ 3’ - ACGAGCCTGCAAAAAAAAAAAAAAAAAAAA - 5’	18585.4 Da

Figure 3

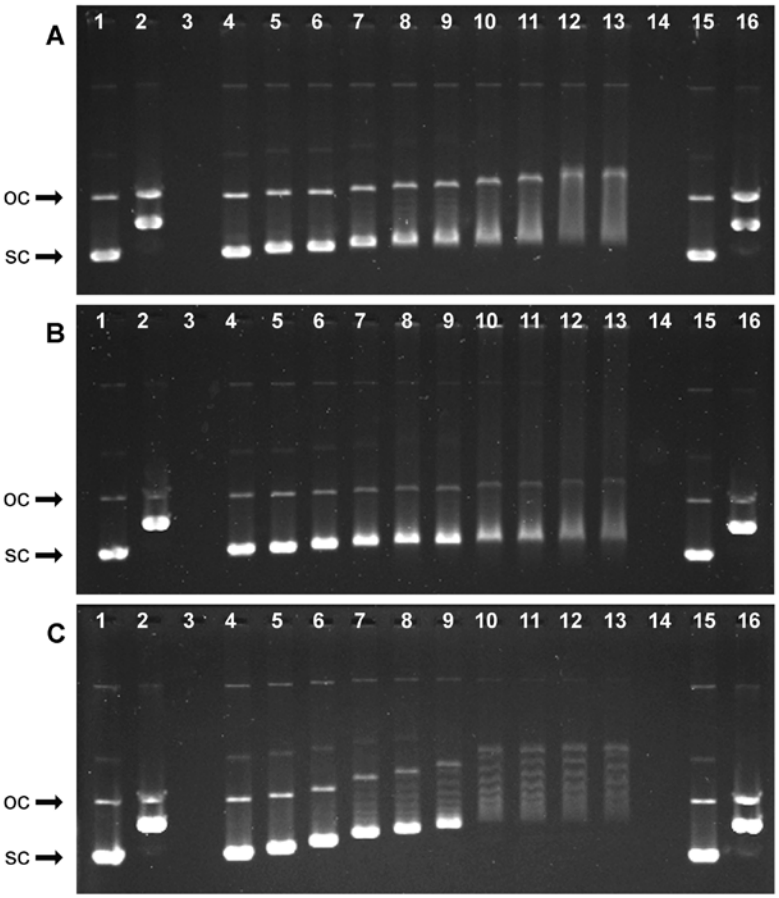
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
OC
SC
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
OC
SC
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
OC
SC

Figure 4

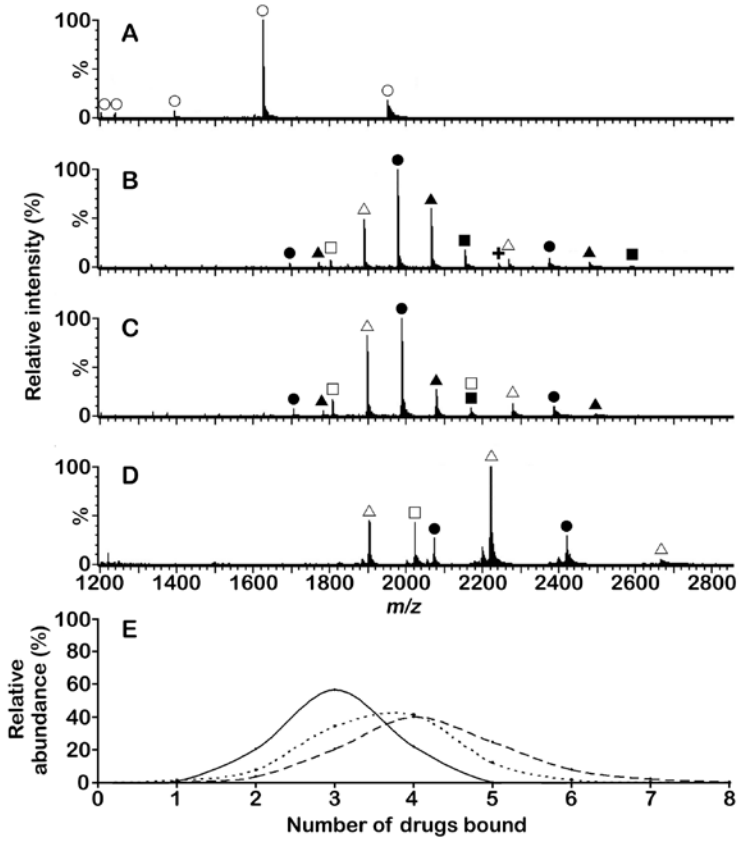
A
B
C
D
E
100
%
0
Relative intensity (%)
1200
1400
1600
1800
2000
2200
2400
2600
2800
m/z
Relative abundance (%)
80
60
40
20
0
1
2
3
4
5
6
7
8
Number of drugs bound

Figure 5

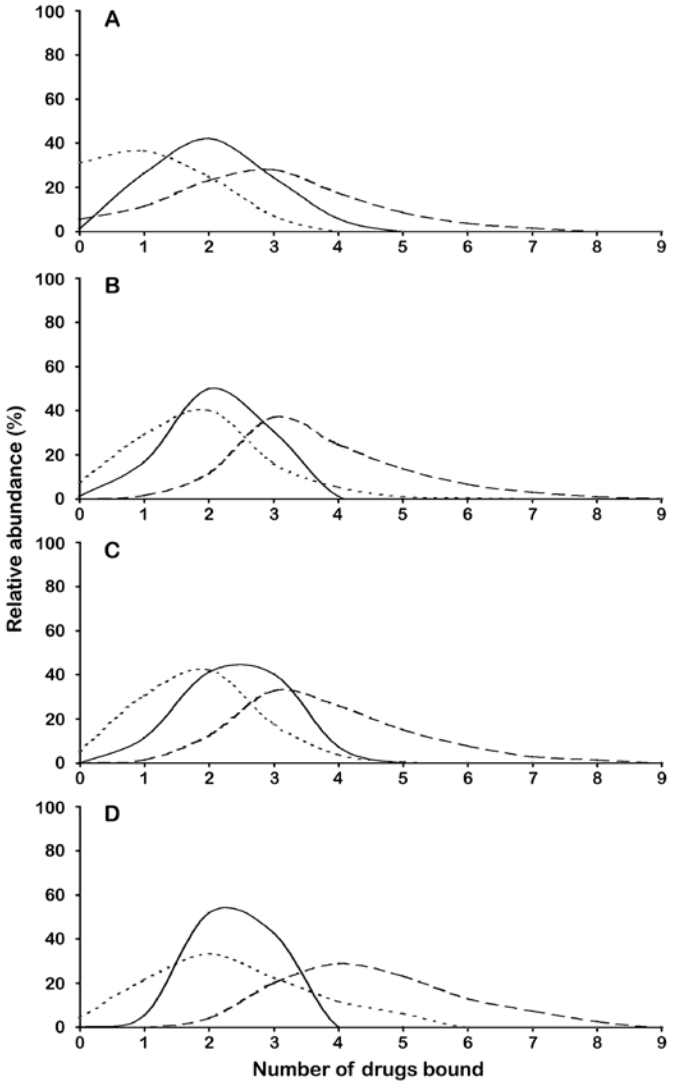
A
B
C
D
Relative abundance (%)
Number of drugs bound
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8 9

Figure 6

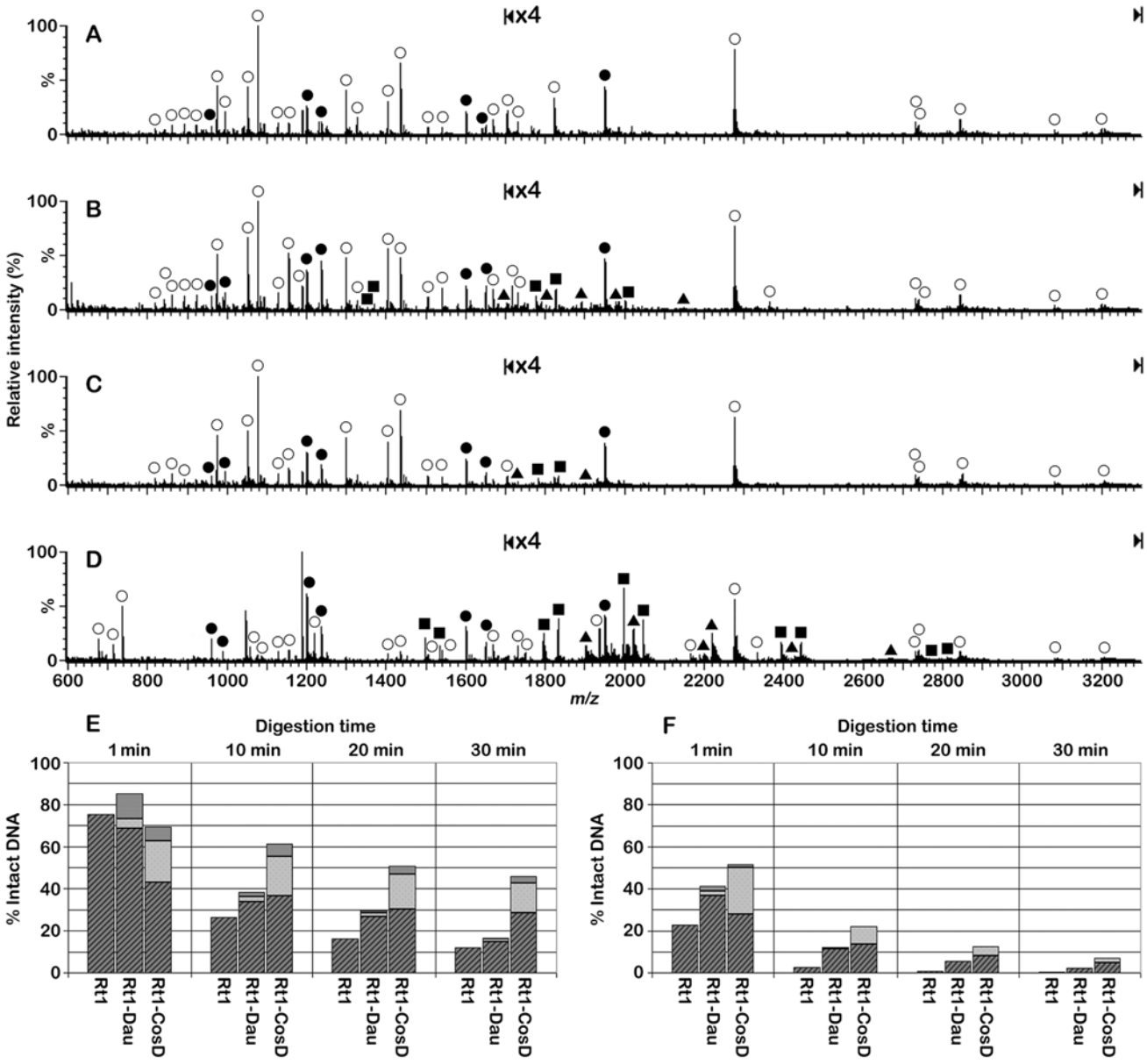
A
B
C
D
x4
100
%
0
Relative intensity (%)
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
3200
m/z
E
F
Digestion time
1 min
10 min
20 min
30 min
% Intact DNA
Rt1
Rt1-Dau
Rt1-CosD

Figure 7