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Role of the amino sugar in the DNA binding of disaccharide anthracyclines: crystal structure of the complex MAR70/d(CGATCG)

Claudia Temperini,[a] Maurizio Cirilli,[b] Massimiliano Aschi[c] and Giovanni Ughetto[d,*]

[a]*Department of Chemistry, University of Florence, 50019 Sesto F.no (FI), Italy*
[b]*Institute of Neurobiology and Molecular Medicine, Rome, Italy*
[c]*Department of Chemistry, Chemical Engineering and Materials, University of l'Aquila, 67010 l'Aquila, Italy*
[d]*Institute of Crystallography, CNR, cp 10, 00016 Monterotondo Stazione, Italy*

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Abstract—Disaccharide anthracyclines analogues have been shown to exhibit different antitumour activity as compared with parents compounds doxorubicin and daunomycin. Here we report the crystal structure of the disaccharide analog MAR70 complexed with the DNA hexamer d(CGATCG). The structure has been solved at 1.54 Å resolution and is similar to previous crystallized anthracycline–DNA complexes with both sugar rings of the disaccharide chain lying in the DNA minor groove. Comparison with the structure of MEN10755 another disaccharide anthracycline co-crystallized with the same DNA hexamer suggests a correlation between the position of the amino sugar on the disaccharide chain and the conformation of this moiety when binding to DNA. This is discussed with respect to the influence on drug activity and on the possible interaction with other cellular targets.

1. Introduction

Anthracyclines are an important class of antitumour drugs and the parent compound doxorubicin has been among the most widely used cancer therapeutic agents for over 30 years.[1] The cytotoxic activity of anthracyclines is generally ascribed as being primarily a result of their interaction with cellular DNA. Like many other antitumour drugs anthracyclines work by DNA intercalation, which results in interfering with the action of topoisomerases, enzymes that govern DNA topology during cellular DNA processes. The effect of such interference is the induction of DNA strand breaks and the formation of a ternary complex drug–DNA–topoisomerase in which the enzyme is covalently linked to the broken DNA strand, the critical event leading to apoptosis and cell death. This is currently the most accepted mechanism for the antitumour activity of these drugs.[2]

Due to their importance the interactions of anthracyclines with DNA have been extensively studied with a variety of biophysical and biochemical techniques and anthracyclines are the best characterized group of DNA intercalators.[3] In the nucleic acid data base there are over 25 crystal structures of anthracycline/DNA complexes which have provided a detailed picture of the interactions between the two molecules and valuable information on structure/function relationship.[4]

Anthracyclines suffer several side effects that limit their use and there has been an intense effort in analog synthesis to obtain better therapeutic drugs. In spite of these efforts the original parent compounds doxorubicin and daunomycin remain the most effective agents of this class.[5] However recently there is a considerable interest in disaccharide anthracyclines. A novel disaccharide analog MEN10755 has been found to show a different activity spectrum as compared to doxorubicin and is active against doxorubicin-resistant tumours.[6] The crystal structure of this drug complexed with the DNA hexamer d(CGATCG) [7] has revealed that the second sugar ring in the disaccharide chain may give rise to two different binding modes. This behaviour is thought to depends on the position of the amino sugar in the disaccharide

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* Corresponding author. Tel.: +39 0690625142; fax: +39 0690672630; e-mail: giovanni.ughetto@ic.cnr.it

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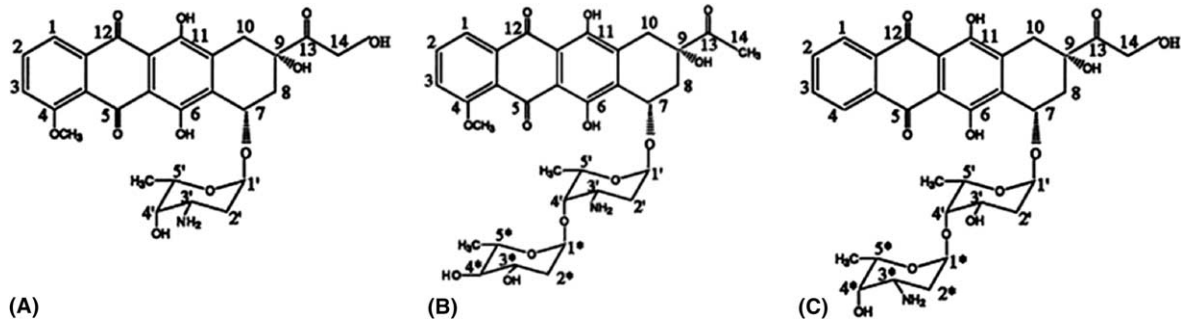

Figure 1. Molecular formula of (A) doxorubicin, (B) MAR70 (4′-(O-L-4′-*epi*-2′-deoxyfucose)-daunomycin) and (C) MEN10755 (3′-deamino-3′-hydroxy-4′-(O-L-daunosaminyl)-4-demethoxydoxorubicin).

chain which may account for the differences in biological activity.

We have studied by X-ray crystallography another disaccharide anthracycline MAR70 which, apart from small differences in the aglycone moiety, differs mainly from MEN10755 for the sugar sequence in the disaccharide chain (Fig. 1). We have co-crystallized MAR70 with the same DNA hexamer d(CGATCG) as MEN10755 and compared the crystal structures. We discuss the results with respect to the importance of the amino sugar in DNA binding and to the possible influence on the interactions with other cellular targets.

2. Results and discussion

2.1. Molecular structure

The overall structure of the complex is very similar to the previous crystallized anthracycline–DNA complexes. The self-complementary hexamer d(CGATCG), that here we denote from C1 to G6, adopts a distorted double helical B-DNA with a MAR70 molecule intercalated between the CpG steps at both ends of the duplex (Fig. 2). The two halves of the complex are symmetrically equivalent and the two binding sites at two ends of the hexamer are identical. The electron density of composite omit map around the binding site is shown in the supplementary materials. The main features that characterize the binding of anthracyclines to DNA are also observed in this structure. The aglycone drug chromophore intercalates between the base pairs in a perpendicular fashion with ring D protruding out into the major groove and both sugars of the disaccharide moiety lying in the minor groove. The O9 hydroxyl group in ring A forms two hydrogen bonds with the N2 amino group and the aromatic N3 atom of the second guanine in the sequence. These hydrogen bonds are responsible for the peculiar orientation of the chromophore in the DNA-intercalation of anthracyclines.

DNA geometry and DNA backbone distortions are also very similar to those observed in the other

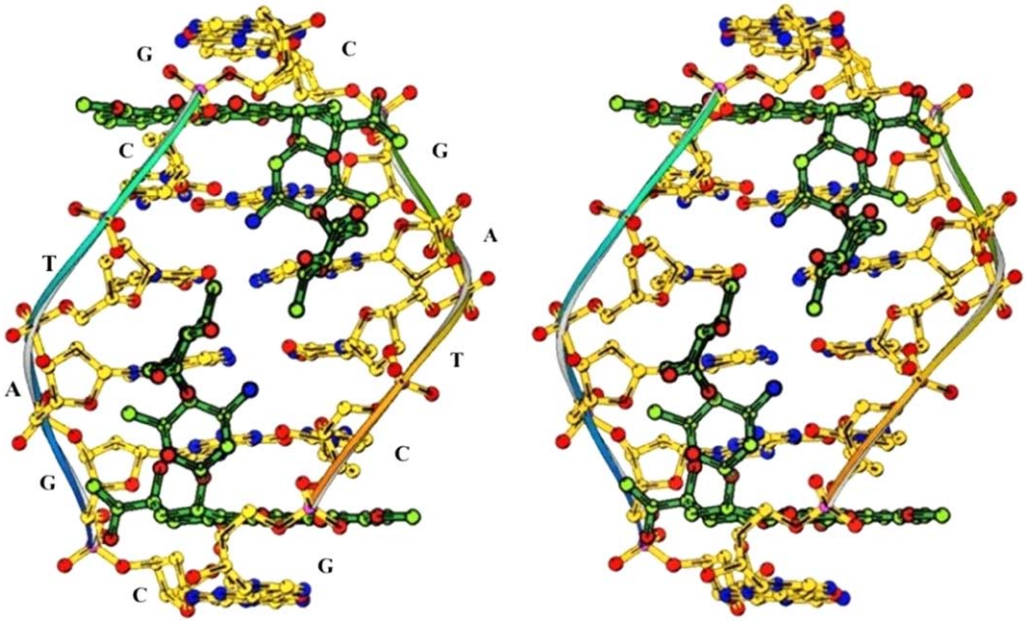

Figure 2. A stereoview of the crystal structure of the MAR70-d(CGATCG) complex. In the DNA molecule the nitrogen atoms are blue, the oxygen atoms are red, the phosphorous atoms are purple and the carbon atoms are yellow. The drug molecule is green. Balls and Stick representation is from Bobscript.[28]

anthracycline–DNA complexes. The conformations of the DNA molecules in this complex and in the doxorubicin–d(CGATCG) complex[8] are almost identical with a root mean square difference of 0.220 Å. The doxorubicin portion of MAR70, excluding the second glycosidic ring and the 14-hydroxyl group in ring A, is also very close to doxorubicin with a rmsd of 0.144 Å (Fig. 3). Torsion angles and geometrical DNA helical parameters are given in the supplementary material.

It has been suggested that the disaccharide moiety lying in the minor groove may induce propagated structural DNA perturbations that may influence the mode of recognition of topoisomerases or other DNA-regulatory proteins. On the contrary, our results suggest that the presence of a second sugar does not affect the binding of the aglycone and of the first sugar to DNA and does not cause significant DNA alterations when compared with the complexes formed by monosaccharide anthracyclines. This is also confirmed by the other two known crystal structure of disaccharide anthracycline–DNA complexes: MAR70/d(CGATCG)[9] and MEN10755/d(CGATCG)[7] (see later).

In the disaccharide chain both sugars are in the chair conformation and fill up the minor groove. Since there are two drug molecules intercalated into one DNA hexamer, the minor groove at the A–T step is shared by both the carbohydrate moieties. However there are no interactions between these moieties as the second sugar rings are facing only the corresponding thymine residue (Fig. 2).

2.2. Comparison of disaccharide anthracycline–DNA complexes

Many di and trisaccharide anthracyclines of natural or synthetic origin were known but none of them showed to possess a better activity than the parent drugs. In natural oligosaccharide anthracyclines the increase in the length of oligosaccharide chain may result in an increase of DNA-binding affinity. However there is no direct correlation between DNA affinity and antitumour activity.[10] MAR70 like other semisynthetic disaccharide anthracycline derivatives did not show a significant antitumour activity comparable to that of the parent drugs. It was found to be active against transplantable leukaemia P388 in mice, yet at a concentration three times higher of doxorubicin.[1]

In contrast, a doxorubicin analog of a novel series of anthracycline disaccharide has been found to exhibit a broader spectrum of antitumour activity in comparison with the parent drugs.[6] Previously we have determined the crystal structure of this analogue MEN10755 complexed with the same DNA hexamer d(CGATCG).[7] It is of interest to compare the structural effect of different drug moieties for the DNA binding in order to see whether such differences may be related to differences in biological activity.

The aglycon moiety of MEN10755 is lacking the 4-methoxy group as the monosaccharide analog idarubicin, while MAR70, like daunomycin, does not bear the 14-hydroxyl group (Fig. 1). However in the structure of the MEN10755 complex, as well as observed here in this structure, the orientation of the intercalated aglycon chromophore and the DNA backbone conformation are very similar to those observed in the other anthracycline–DNA complexes (Fig. 2). Thus it is apparent that small differences on the aglycone chromophore and the presence of a second sugar in the saccharide moiety does not affect the stacking pattern of anthracyclines to any significant extent.

On the contrary, major structural variations between these two structures can be noted in the conformation of the sugar chains. In the MAR70 complex both sugar rings lie in the minor groove spanning four base pairs. In the MEN10755 complex there are two different conformations of the sugar moiety resulting in two different

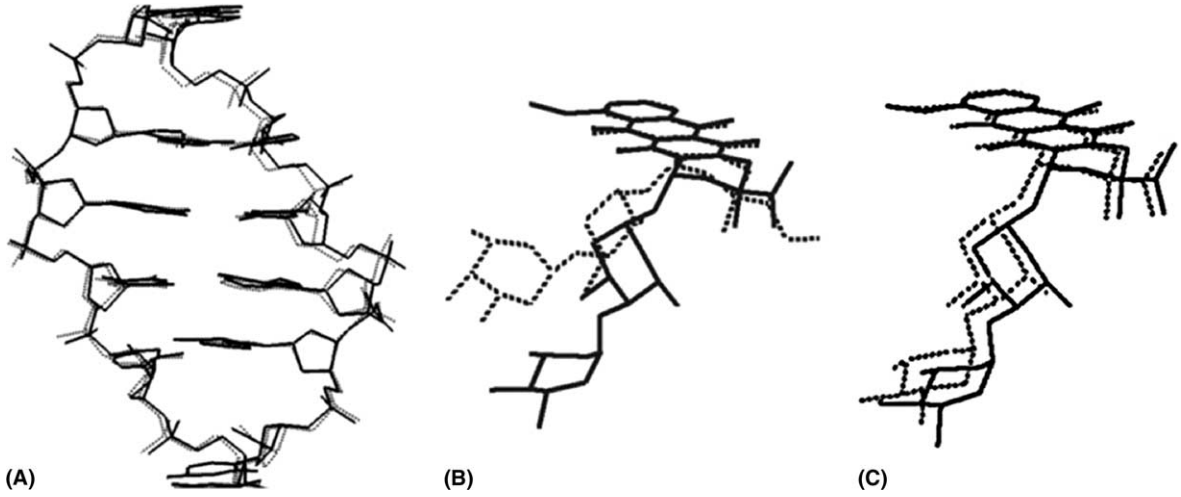

Figure 3. (A) Comparison of DNA conformations in MAR70-d(CGATCG) (filled lines) and MEN10755-d(CGATCG) (dashed lines) complexes. The DNA molecules are superimposed by least square fitting of the first (cytosine) and in the last (guanosine) bases in the sequence (left). (B) Comparison of the conformation of MAR70 (filled lines) in this complex, with that of MEN10755 (dashed lines) in the second binding site and (C) comparison of MAR70 conformation in this complex (filled lines) and the complex with d(CGATCG).[7] The drug molecules are superimposed by least square fitting of the common atoms on the chromophore.

binding sites. In one site the conformation is similar to that observed here with the carbohydrate moiety lying in the minor groove. In the other site the first sugar is shifted towards the phosphate group at the GpC step and the second sugar protrudes definitely out from the minor groove.

The different binding modes of the carbohydrate moiety of the two drugs observed in the two complexes do not produce any significant difference in DNA conformation. We can see in Figure 3A that the DNA molecules in the two structures are very similar with a root-mean-square difference 0.564 Å.

The position of the amino sugar in the disaccharide chain is the main feature that characterizes the two drugs. In all amino glycosides from natural origin the amino sugar is the first moiety directly linked to the aglycone moiety.[1] In MAR70 the first sugar in the carbohydrated chain is daunosamine, like in the parent compounds daunomycin and doxorubicin; the second sugar linked via α(1–4) to the fist sugar is 4′-*epi*-2′-deoxyfucose. The doxorubicin disaccharide analog, MEN10755, has the first sugar without the 3′-amino group, substituted for a hydroxyl group, and the second sugar, daunosamine, linked via α(1–4) to the first sugar. In both analogs the linkage between the two sugars is in the axial orientation as this configuration has been shown to be a structural determinant for the activity of disaccharide anthracyclines.[11]

There is a conformation variability in the glycosyl linkage observed in the disaccharide chain of the two complexes. However in both complexes the torsion angles in the glycosyl linkage between the first and the second sugar though slightly different are such that they make the two rings nearly perpendicular each other. This conformation is one of the most frequently observed in disaccharide structures and has been observed also in the crystal structure of the complex of MAR70 with d(CGTACG).[9] More important for the orientation of the disaccharide chain are the torsion angles C8–C7–O7–C1′ and C7–O7–C1′–C2′ between the aglycon and the first sugar. In this complex they are 84° and 138°, very close to those observed in the MAR70/d(CGATCG) complex. Indeed the conformations of MAR70 in both complexes is almost identical (Fig. 3C). In MEN10755 complex at the site where the second sugar protrudes outside the minor groove these torsion angles are 103° and 165°, respectively. These small differences are sufficient to drive away from the minor groove the first sugar and to affect dramatically the orientation of the second sugar (Fig. 3B). As a result of this different conformation in the MAR70 complex the N3′ substituent on the first sugar is at hydrogen bond distance 3.1 Å from O2 of the C5 cytosine; in MEN10755 complex the distance between the hydroxyl on the same 3′ position is at 4.6 Å from O2 of the C5 cytosine residue and a tetracoordinate water molecule occupies the cavity created by the shifting the first sugar. The torsion angles in the glycosyl linkage observed in the crystal structures of DNA/disaccharide anthracycline complexes are summarized in the supplementary material.

2.3. Role of the amino sugar

In the crystallized complexes of monosaccharide anthracyclines the sugar moiety lies in the minor groove and the orientation and the interactions with DNA vary from structure to structure depending on DNA sequence. However the sugar ring fills up the minor groove and the charged amino group at the 3′ position has always been found to approach the edges of base pairs leaving no room for solvent molecules. In the case we are comparing here the two complexes are formed with the same DNA sequence so the differences observed in the conformation of the sugar chain can depend only on the presence of the charged amino group on the first sugar.

The amino sugar in this position seems to dictate the presence of an A–T or T–A base pairs adjacent to the intercalation site and to be a determinant of the DNA sequence specificity of anthracyclines. This sequence preference is consistent with the sequence specificity of topoisomerase II-stimulated DNA cleavage and is included in the DNA hexamer of the two complexes.[12] Therefore the different conformations that the two analogs have been found to adopt may be a critical factor in the stabilization of the ternary complex and may be of importance for the interactions in the active site.

To accomodate the drug chromophore at the intercalation site a local DNA unwinding is produced. The electrostatic potential of the solvent accessible surface in the cavity created in the minor groove of the unwound DNA is less negative than in the regular B DNA. However in the region where lies the first sugar ring a considerably negative surface is still present. In Figure 4 this electrostatic potential surface is represented for the d(CGATCG) sequence of both complexes with MAR70 and MEN10755. We can see that a negative charged potential faces the 3′ position of the sugar, which in the MAR70 molecule is a charged amino group and in MEN10755 molecule is a neutral hydroxyl group.

The free energy contributions of various functional groups of doxorubicin have been analyzed by several computing methods and compared with experimental binding data. In computational studies the presence of a positively charged amino group at the 3′ position on the sugar ring has been shown to give a relevant contribution.[13,14] The electrostatic contribution to the free energy for several anthracycline ligands have been calculated and a very significant free energy difference has been seen associated with modification of the N3′ amino to a hydroxyl group in agreement with experimental data.[15,16]

The computational analyses of DNA binding have been performed only for monosaccharide anthracyclines. The complexes we have studied are formed by a DNA hexamer with two intercalated drugs. The disaccharide moiety, especially the second ring, shares the interactions with the third and fourth base pairs. The effect we have observed in the complex with MEN10755 where one of

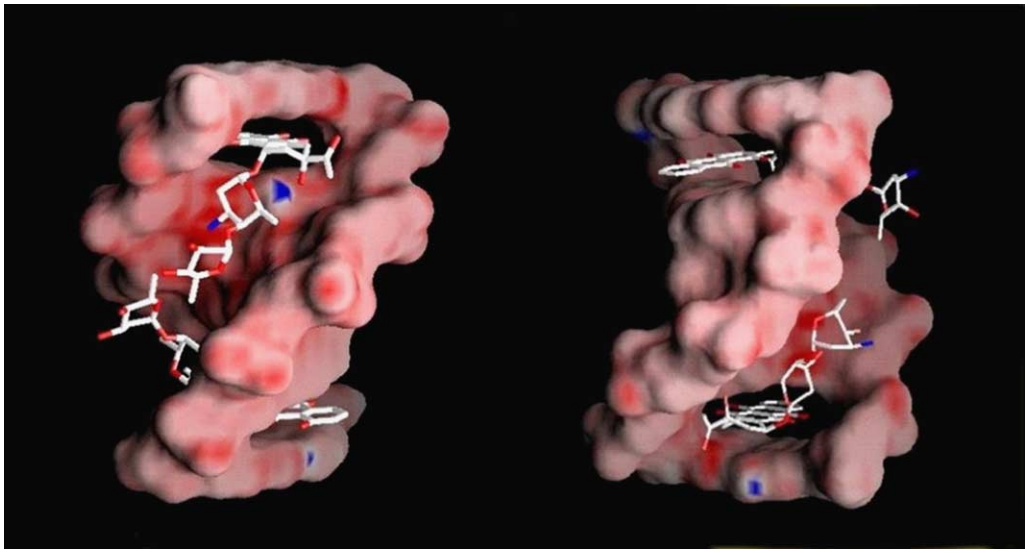

Figure 4. Distribution of the electrostatic potential of the DNA molecule: (left) in the MAR70-d(CGATCG) complex, (right) in the MEN10755-d(CGATCG) complex. The red colour represents a negative value, white colour a zero value and blue colour a positive value. Figure generated with the GRASP Software.[29]

the second sugar rings is flipped away from the minor groove may be caused by this crowded situation.

We have calculated the binding free energy of the disaccharide anthracyclines MEN10755 and MAR70 with the DNA sequence formed by the quartet CGAT. The coordinates of the crystal structures were used for the model, taking only one of the two drug molecules intercalated into the DNA hexamer and the four base pairs of the drug binding site. In the case of the MEN10755 complex the binding site with both sugar rings lying in the minor groove was chosen.

Our results with the two drugs binding the same DNA sequence show a binding free energy of −11.7 kcal/mol for MAR70, −8.7 including the solvent effect, and −8.3 (−7.3) kcal/mol for MEN10755. The substitution for the amino group by a hydroxyl group, while reducing the free binding energy of MAR70, does not affect significantly that of MEN10755 where the amino group is on the second sugar. Our calculations actually do provide only an estimation of the binding free energies, however they are comparable with the other computational tools utilized to analyze the DNA binding of monosaccharide anthracyclines.[16]

As we have considered the same DNA quartet CGAT, there are no sequence-dependent effects and the results confirm that the contribution of the amino group on the first sugar is the structural determinant of the interactions of the DNA binding of disaccharide anthracyclines. The loss of the electrostatic contribution of the charged amino group to the binding free energy allows the first sugar linked to the aglycon to flip a little away from the the minor groove enough to affect significantly the orientation of a bulky substituent or of a second sugar. Indeed previous studies have shown that disaccharide analogs lacking the amino group on the first sugar can stimulate topoisomerase I-mediated DNA cleavage though at a lower level than topoisomerase II.[17] On the other hand the analog 3′-morpholinyl-doxorubicin, bearing a bulky substituent on the no longer charged 3′ position, has been found to interfere with topoisomerase I but not with topoisomerase II.[18]

3. Conclusions

The results of the present study and the comparison of the two complexes formed by MAR70 and MEN10755 with the same DNA sequence confirm that the presence of a second sugar in disaccharide anthracyclines does not influence their mode of intercalation to DNA.

The orientation and the interactions with the DNA molecule of the aglycone moiety are almost identical to those observed in monosaccharide anthracycline/DNA complexes. Also the bulky 4-methoxy group on ring D of the chromophore has no effect on the intercalation geometry in agreement with previous structural studies on monosaccharide anthracyclines. In the MAR70 complex, where the 4-methoxy group is present, and in the MEN10755 complex lacking this group, the intercalation geometry is almost identical. The influence on drug activity by the presence on the aglycone moiety of this substituent, which protrudes out into the major groove, may be the result of interactions with other proteins, as it may occur in the ternary complex drug–DNA–topoisomerase.

It appears that the differences in biological activity between MEN10755 and MAR70 depend on a different DNA binding of the sugar moiety. In MEN10755 the second sugar ring which is no longer in the minor groove is more likely to interact with other cellular targets. It may result in a more stable or unstable interaction of the drug in the ternary complex. The capability of the second sugar of protruding out of the minor groove is

related to the position of the amino sugar. If daunosamine is the first sugar linked to the aglycon, as in the MAR70 complex, the basic 3′ amino group contributes an electrostatic interaction to the binding free energy. This additional interaction further stabilizes the position of the first sugar which lies deeply in the minor groove and makes it difficult for the second sugar to be in contact with other than the DNA or the solvent molecules. In monosaccharide anthracyclines it has been shown that the substitution of the basic amino group at the 3′ position for a hydroxyl group results in reduced DNA-binding affinity but it is not an essential determinant for drug activity. Analogs bearing this modification retain a significant cytotoxic and antitumour activity comparable with that of parents compounds.[9] Disaccharide anthracyclines with a daunosamine as first sugar could enhance DNA binding affinity but should not change the mode of binding with the sugar chain lying in the minor groove spanning more than three base pairs. Disaccharide analogs lacking a basic amino group at the 3′ position in the first sugar are more flexible and the second sugar may not participate to the DNA binding and instead influence external interactions. These interactions could play an important role in the formation of the ternary complex drug–DNA–topoisomerase and may affect the ability of the drug to poisoning both topoisomerase I and II. The interactions with other cellular targets involved in multidrug resistance may also be affected. Our results are relevant to provide further information on the critical role played by the amino sugar as a structural determinant to modulate antitumour activity of disaccharide anthracyclines.

4. Materials and methods

The self-complementary DNA hexamer d(CGATCG) was synthesized on an Applied Biosystem plus DNA synthesizer using phosphoramidite methodology.[19] After cleavage from the resin, the crude material was deprotected and purified by reverse-phase HPLC on Dynamax Pure DNA column, 21.4 mm × 5 cm, 30.0 nm (Rainin). MAR70 was supplied by Dr. F. Arcamone (Farmitalia). Crystals were grown at room temperature in sitting drops, using a vapour diffusion technique. Red crystals appear within 5 weeks from a solution of 20 mM sodium cacodylate, pH 6.0; 1.5 mM DNA, 9 mM $MgCl_2$, 1.5 mM spermine hydrochloride, 1.5 mM Mar70 and 10% (by vol.) 2,4-methyl-pentanediol (MPD) equilibrated against a reservoir containing a solution of 35% MPD.

The crystals were mounted in a sealed glass capillary with a droplet of mother liquid. Diffraction data were collected on a Rigaku AFC5R four circle diffractometer using a Rigaku RU200 rotating anode, operating at 50 V, 120 mA, in ω scan mode with CuKα radiation (1.5418 Å). The unit cell dimensions were determined to be $a = b = 28.19$ Å, $c = 53.25$ Å in the tetragonal space group P41212. Data were collected and processed using the TEXSAN program.[20] A total of 3484 unique reflections were obtained with a completeness of 87.0% to a resolution of 1.54 Å. The structure was solved by the molecular replacement methods with A.Mo.Re,[21] using as starting model the coordinates of he complex doxorubicin/d(CGATCG),[8] and refined with the SHELX-97 package.[22] Electron density maps ($2F_o - F_c$) and ($F_o - F_c$) were calculated with CNS program[23] and displayed using the graphic program O.[24] The final model had an *R*-factor of 18.0%, *R*-free 23.4%, for 2081 reflections at $F > 4\sigma(F_o)$ in the resolution range 10–1.54 Å with a rms deviation from standard geometry of 0.013 Å in bond lengths and 0.038 Å in bond angles. In the asymmetric unit there are one DNA strand, one drug molecule and 21 solvent molecules. Crystallographic parameters and refinement statistics are summarized in Table 1.

Table 1. Crystallographic parameters and refinement statistics

Parameter	Value
X-ray source	Rotating anode
Wavelength (Å)	1.54
Cell parameters	$a = b =$ 28.19 Å c = 53.25 Å $\alpha = \beta = \gamma = 90°$
Space group	$P4_12_12$
No. of unique reflections	3484
Completeness (%)	87.0
No. of reflections [$>4\sigma(F_o)$]	2081
$\langle I/\sigma(I)\rangle$	4.1
Resolution range (Å)	10–1.54
R-factor (%)	18.0 (24.9)[b]
R-free (%)[a]	23.4
Rmsd of bonds from ideality (Å)	0.013
Rmsd of angles from ideality (Å)	0.038

[a] Calculated using 5% of data.
[b] Values in parenthesis are for last resolution shell (1.77–1.54 Å).

The calculations of the binding free energies were performed using the program package GAMESS.[25] Quantum chemical calculations at the Hartree–Fock level of theory with a 3-21G basis set[26] were carried out with the use of the Polarizable Continuum Model (PCM)[27] in order to estimate the binding free energies in aqueous solution. The protonation states at the physiological conditions were imposed for the amino and phosphonate groups. The complex as well as the single partners were geometrically relaxed in the gas phase obtaining the gas-phase binding free energies (DG1 and DG2) then the solvation free energies were calculated using the PCM approach and finally the related free energies in aqueous solution (DGaq) were evaluated.

The coordinates and structure factors have been deposited with the Nucleic Acid Database: NBD ID DD0061.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2004.12.007.

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