Mitomycin C binding to Poly[d(G-m⁵C)]

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Poly[d(G-m⁵C)] was modified by reductively activated mitomycin C, an anti-tumour drug, under buffer conditions which are known to favour either the B or the Z conformations of DNA. C.d. and ³¹P-n.m.r. were used to characterize the poly[d(G-m⁵C)]-mitomycin cross-linked complexes, as well as the effects on the equilibrium between the B and Z forms of the polynucleotide. Mitomycin C appears to inhibit the B → Z transition,

even in the presence of 3 mM MgCl₂, while the Z-form of poly[d(G-m⁵C)] does not interact significantly with the drug under bifunctionally activating conditions; thus no reversion from the Z-form to the B-form of the polynucleotide can be observed under the salt conditions which are required for the Z-form to exist.

INTRODUCTION

Mitomycin C is widely used in the treatment of a variety of tumours [1,2]. Among its most interesting properties is the ability to bind covalently to DNA, resulting in the formation of cross-links between the complementary strands through bifunctional alkylation [3–7]. Mitomycin C (Figure 1) is peculiar in that it needs a reductive activation to react with DNA. The enzymic or chemical reduction induces an opening of the aziridine ring of the molecule. Mitomycin is known to bind to DNA with cross-link formation favoured over monoalkylation on reductive activation with sodium dithionite [7]. A striking feature is that the drug is covalently linked exclusively to guanine residues. The bases are linked at their N-2 positions to mitomycin at its C-1 and C-10 atoms [6]. Numerous results suggest that the cytotoxic action of mitomycin is related to its capacity to interact with cellular DNA. Even though the formation of cross-linked adducts has been described as the cause of the lethal effect on the transformed cells, recent studies indicate that some chemical derivatives of mitomycin which are incapable of forming DNA crosslinks also present anti-tumour effects [8].

DNA regions containing CpG sequences, and thus potential sites for the formation of mitomycin adducts, have been shown to be associated with many tissue-specific genes and may represent regulatory regions in several of these genes [9]. There are a number of reasons for believing that alterations in the pattern of methylation (m⁵C) in CpG tracts are related to the activation/inhibition of cellular growth and could also promote the carcinogenic process. The conversion of DNA from the B to the Z conformation has been studied in many systems [10], and it has been shown that methylated CpG regions can favour the presence of Z-DNA under biological conditions [10,11]. In fact, the level of transcription of the *c-myc* oncogene is unambiguously associated with the formation of Z-DNA in discrete regions of the gene [11], and it is, therefore, of interest to determine whether mitomycin and other drugs involved in cancer therapeutics might interfere with the B → Z transition in DNA. In general, this appears to be the case [12–15], but the precise characteristics of the mechanism involved are far from being completely understood. It is of interest to study the mitomycin C interaction with the B and Z forms of DNA, since it is possible to obtain adducts of irreversible bifunctionately cross-linked drug by using well-determined experimental techniques [7], while the use of

poly[d(G-m⁵C)] ensures that the conformational transitions take place at physiological ionic strength. On the other hand, the effect of mitomycin C on the B → Z transition was previously characterized using other polynucleotides, producing misleading results [7,16]. At present, it seems fully accepted that mitomycin C binds to B-DNA but not to Z-DNA [7,17,18], while the inversion of the c.d. spectra in the presence of cross-linked mitomycin (see the Z-like spectra in Figure 2a) can be due to induced c.d. effects as a result of electronic interaction between the bases and the cross-linked drug [17].

In the present study we analysed the equilibrium between B and Z forms of poly[d(G-m⁵C)] and the effect exerted by the bis-adducts of mitomycin C on m⁵CpG bases by using ³¹P-n.m.r. and c.d. spectroscopic approaches on mitomycin cross-linked to poly[d(G-m⁵C)].

MATERIALS AND METHODS

Poly[d(G-m⁵C)] was purchased from Pharmacia-Iberica. Mitomycin C was obtained from Boehringer Mannheim. Poly[d(G-m⁵C)] was dissolved by rotational mixing at 4 °C for about 16 h in 25 mM Pipes (pH 7.0)/50 mM NaCl (Buffer B). When required, MgCl₂ was added to 3 mM (Buffer Z) in order to obtain the Z-conformation of the polynucleotide. All the samples were dialysed against a large volume of the required buffer, and sonicated under a nitrogen stream (2 min at 0 °C) in order to reduce the viscosity.

Treatment of poly[d(G-m⁵C)] with mitomycin C under reductive bifunctional activating conditions

A solution containing 3.5 mM (in nucleotides) of sonicated poly[d(G-m⁵C)] and different amounts of a mitomycin C stock solution were mixed to obtain different ratios of added mitomycin C to nucleotide between 0.5 and 3 in either Buffer B or Buffer Z, and deaerated by purging with both nitrogen and argon. Sodium dithionite (Na₂S₂O₄) was added to the mixture as described elsewhere [7]. When required, separation of unbound mitomycin was accomplished by dialysis [4] against Buffer B, or Buffer Z for experiments performed under Z-forming conditions.

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Figure 1 Molecular structure of mitomycin C

Determination of the mitomycin C to nucleotide-binding ratios

Binding ratios were determined as described in [19]. An ϵ_{254} value of $8400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (nucleotide) $\cdot \text{cm}^{-1}$ for poly[d(G-m⁵C)] and an ϵ_{314} of $11000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for bound drug were used. Table 1

displays the mitomycin C to nucleotide-binding ratio for some of the complexes used.

C.d. measurements

C.d. measurements were performed in 1- and 0.05-cm-path-length cells (the latter cells allowed us to measure c.d. spectra of samples taken directly from n.m.r. tubes) using a Jasco J-720 spectropolarimeter interfaced to a Hewlett-Packard Vectra 386/25 computer. The temperature was kept constant (25 °C). The wavelengths scanned ranged from 220 to 400 nm. Scans were recorded at 1 nm intervals, and two spectra of each sample were recorded and averaged. A scan of buffer alone was recorded to provide a baseline. Molar ellipticities $\{[\theta] (\text{M}^{-1} \cdot \text{cm}^{-1})\}$ were calculated per nucleotide residue.

³¹P-n.m.r. spectroscopy

³¹P n.m.r. was registered with a Varian Unity 300 (³¹P, 121.4 MHz) equipped with an HFPC four nucleus probe and using special low-volume n.m.r. tubes with bulk magnetic sus-

Figure 2 Effects of bifunctionally activated mitomycin C on the c.d. of poly[d(G-m⁵C)]

(a) C.d. of the polynucleotide (—) in Buffer B (25 mM Pipes (pH 7.0)/50 mM NaCl) and in the presence of reductively activated mitomycin C at the following ratios of added drug to nucleotide: 0.5 (.....); 1 (— · — · —); and 3 (— — —). (b) C.d. of the polynucleotide (—) in Buffer Z [25 mM Pipes (pH 7.0)/50 mM NaCl/3 mM MgCl₂] and in the presence of mitomycin C at the following ratios of added drug to nucleotide: 0.5 (.....); 1 (— · — · —); and 3 (— — —).

Table 1 Preparation and some characteristics of the Poly[d(G-m⁵C)]–mitomycin C complexes used in the experiments

Sample	Molar ratio (r_a) (mol of drug added/ mol of nucleotide)	Binding ratio (r_b) (mol of drug/ mol of nucleotide)	Conformation indicated by:	
			C.d. spectrum	³¹ P-n.m.r.
Poly[d(G-m ⁵ C)] (B-form) plus mitomycin C, in Buffer B*	3	0.18	B	B
Poly[d(G-m ⁵ C)] (B-form) plus mitomycin C, in Buffer Z†	3	0.12	Z-like‡	B
Poly[d(G-m ⁵ C)] (Z-form) plus mitomycin C, in Buffer B	3	0.015	B	B
Poly[d(G-m ⁵ C)] (Z-form) plus mitomycin C, in Buffer Z	3	0.040	Z	Z

* Buffer B, 25 mM Pipes (pH 7.0)/50 mM NaCl.

† Buffer Z, 25 mM Pipes (pH 7.0)/50 mM NaCl/3 mM MgCl₂.

‡ This spectrum might be due to a drug-induced c.d. spectrum (see the text).

ceptibility equal to those for solvent (water/²H₂O, 1:10). The samples of the polynucleotide solution in either B or Z conformation and the corresponding amount of added mitomycin (about 0.30 ml final volume) were flushed with argon to avoid the interference of oxygen with sodium dithionite. The main acquisition and processing parameters were as follows: pulse width, 50°, acquisition time = 1 s, recycle time = 1.5 s, spectral window = 3000 Hz, Waltz ¹H decoupling, line broadening = 5 Hz and Fourier transform points = 16384. All spectra were acquired overnight (number of transitions = 40000) at 25 °C. Chemical shifts (δ) are given in p.p.m. and referenced externally against trimethyl phosphate acquired in the same conditions as the sample (Z0, lock field and shim values) to avoid inconsistencies in the reference.

RESULTS

C.d. studies

Figure 2 shows the effect of adding different amounts of mitomycin C to poly[d(G-m⁵C)] under bifunctional activating conditions. Figure 2(a) shows a typical c.d. spectrum of the B-form of poly[d(G-m⁵C)] corresponding to the polynucleotide in the absence of mitomycin, as well as the changes induced by the drug at the added drug/nucleotide ratios indicated in the legend to the Figure. At low added drug/DNA ratios ($r_a < 1$) we observe, in the 240–310 nm region, a B-form of poly[d(G-m⁵C)] without a change in the c.d. spectra produced by the adduct-induced c.d. which is superimposed on the c.d. of the polymer as described for other DNAs [5,16,18], while at quite larger ratios of added drug to DNA ($r_a > 1$) the adduct-induced c.d. phenomenon becomes evident. This is characterized by a Z-like c.d. spectrum [17]. In the 330–400 nm region, Figure 2, under both B- and Z-buffer experimental conditions, the polynucleotide shows no c.d. signal. In the presence of increased amounts of the reduced mitomycin an extrinsic induced effect is observed in this region outside the c.d. spectral range of poly[d(G-m⁵C)]. Although it is acknowledged that these positive bands outside the polynucleotide region appear to reflect the presence of bound ligand molecules [20], this interpretation is not so obvious for mitomycin C, since at the region around 380 nm the drug alone produces a clear c.d. band of relatively large magnitude [5]. The presence of an isodichroic point at 373 nm (see Figure 2b) might indicate that the DNA conformation changes somewhat after drug binding. However, the binding to the Z-form of poly[d(G-m⁵C)] described through the changes in the molar ellipticity in the 370–380 nm region must be evaluated with caution. These changes resemble those displayed by the mitomycin–B-form complex shown in Figure 2(a), indicating that the drug is also associated in a non-covalent way to some regions in both poly[d(G-m⁵C)] conformations, but only cross-linked to a few regions which might remain in the B-form in the otherwise Z-form polymer. It is known that mitomycin does not bind, for example, to poly[d(G-B^rC)] in the Z conformation [5], and cytosine methylation could enhance mitomycin C cross-linking in DNAs which are in the B conformation [21]. In any case, no symptoms of a Z → B reversion induced by mitomycin are observed in the 210–300 nm region in Figure 2(b).

³¹P-n.m.r. studies

To gain new insight into the meaning of the positive molar-ellipticity changes at the 380 nm region and the Z plus mitomycin spectra, as well as to check whether the drug could bind to the Z conformer of poly[d(G-m⁵C)], we followed a different approach using ³¹P n.m.r. This technique is also helpful to confirm

unambiguously whether the Z-like spectra in Figure 2(a) are due to Z-DNA or to the induced mitomycin c.d. spectrum [17]. It is well known that ³¹P n.m.r. is a sensitive probe of the structure and dynamics of DNA fragments in solution [22]. An important aspect is the sensitivity of phosphorous chemical shift to the conformation of phosphodiester backbone [23]. The B and Z conformers can easily be distinguished [24], and thus any conformational change after cross-linking mitomycin C can be unambiguously assigned to changes in the polynucleotides since mitomycin lacks phosphorous atoms (see Figure 1). First of all, we determined the chemical shifts of the sonicated poly[d(G-m⁵C)] sample in the B and Z conformations (see Figures 3a and 3c). In all cases broad signals appeared, mainly due to the transverse slow motion of DNA fragments in water solution and the broad range of DNA fragments produced by sonication (about 150 bp long [24]) that produce similar, but not identical, resonances. In the absence of magnesium (B-form), only one signal appeared, centred at –4.25 p.p.m. (linewidth around 50 Hz), whereas in the presence of 3 mM MgCl₂ (Z-form) two signals appeared at –4.30 and –2.84 p.p.m. (linewidth around 100 Hz). These two resonances agree with those described previously for Z-DNA [24] and can be assigned to the non-equivalent g,g+ and t,g+ phosphodiester conformations in the left-handed polymer [25].

New n.m.r. signals appeared when mitomycin was added to poly[d(G-m⁵C)] under reductive bifunctional activating conditions, at –1.56 p.p.m. in the case of the B-formation (Figure 3b), and at –1.53 and –0.33 p.p.m. in Buffer Z (Figure 3d). These experimental facts are consistent with the results obtained using other DNA cross-linking agents which, when associated to DNA, make a downfield change in chemical shift of about 2 p.p.m. [26]. However, we observe these changes as narrow singlets, while it is commonly accepted that the presence of bonded drugs restricts the mobility of the DNA strands inducing a notable increase in the linewidth [27]. At first glance the presence of those sharp signals suggests that the drug which is not cross-linked to the polynucleotide can interact through the minor groove. This interaction produces a reversible complex which might locally modify the phosphodiester backbone, leading to similar local structures in both DNA conformers. Mitomycin C shows a rather low affinity for DNA [28], and the drug added to excess (Figure 3 and Table 1) must be involved in a continuous exchange between bound and free ligand. It generates an additional mechanism of relaxation at a few phosphorus atoms which are close to the interacting sites in the minor groove of poly[d(G-m⁵C)], thus rendering these sharp n.m.r. signals. The presence of one additional signal under B-buffer conditions and two under Z-form conditions (Figure 3) indicates that the drug does not seem to induce large conformational changes in the phosphodiester backbone. These well-defined signals also indicate that the binding sites are well located, and there are no transverse or rotational degrees of freedom. Another feature of the n.m.r. spectra in Figures 3 and 5 (below) that merits further consideration is the relative areas of the narrow and broad signals under B- and Z-form conditions. Assuming that the relative relaxation times of the two DNA conformers were similar, and, therefore, the area under each peak was proportional to the number of nuclei that originate the signal, the n.m.r. spectra would indicate that the amount of cross-linked sites in B-DNA is larger than for Z-DNA. The presence of mitomycin binding to B-form poly[d(G-m⁵C)] clearly does not produce a transition to Z-form DNA (Figure 3b), despite the characteristics of the c.d. spectra in Figure 2(a). The changes in the c.d. spectra might reflect the interaction between the chromophores of mitomycin C and DNA through a mechanism similar to that

Figure 3 Effects of bifunctionally activated mitomycin C on the ^{31}P n.m.r. of sonicated poly[d(G-m 5 C)]

(a) ^{31}P -n.m.r. spectrum of the B-form of the polynucleotide. (b) ^{31}P -n.m.r. spectrum of the polynucleotide in the presence of bifunctionally activated mitomycin C ($r_a = 3$). (c) ^{31}P -n.m.r. spectrum of the Z-form of the polynucleotide. (d) ^{31}P -n.m.r. spectrum of the Z-form of the polynucleotide in the presence of bifunctionally activated mitomycin C ($r_a = 3$).

originating the c.d. spectra of DNA by base-base exciton coupling.

Non-covalent and covalent binding of mitomycin C

The results of the mitomycin effect on poly[d(G-m 5 C)] which had been equilibrated previously in the Z-form merit further consideration, since it is noteworthy that the c.d. and n.m.r. results (Figures 2b and 3d) might indicate that mitomycin C binds to poly[d(G-m 5 C)] in the Z-conformation. However, it may be the case that it only binds to the regions which remain in B-form, perhaps in an extension similar to the case of the polymer in the B-conformation, but does not induce a detectable reversion to the B-form. At first glance, the positive ellipticity in the c.d. spectra around 370 nm, discussed above, and the appearance of phosphorus n.m.r. signals at -0.33 and -1.53 p.p.m. (Figure 3) seem to confirm such a hypothesis. However, the experiments displayed in Figures 2 and 3 were performed using samples obtained by simply mixing the polynucleotide and drug under the reductive bifunctional activating conditions described in [7], so at this stage of the work no attempt was made to separate the unbound drug. Therefore, in order to gain further insight into the mitomycin effects on poly[d(G-m 5 C)] the samples used for the n.m.r. experiments (displayed in Figures 3b and 3d) were

divided afterwards in two parts to confirm whether the results discussed above could be a symptom of mitomycin binding to both forms of the polynucleotide or rather due to the presence of unbound drug. The different samples, four in total, were dialysed against either Buffer B or Buffer Z respectively and their drug/nucleotide binding ratios measured (see Table 1) and analysed again using c.d. and ^{31}P n.m.r.

When half of the sample of B-form polynucleotide plus mitomycin (shown in Figure 3b) was dialysed against Buffer B, the resulting c.d. spectrum was of a B-like form (Figure 4a, line 1), while the other half dialysed against Buffer Z displayed some characteristics of the Z-DNA spectrum in the 260–280 nm region (line 2 in Figure 4a) though with negative ellipticity in the 250 nm region (a characteristic of the B-form spectrum). This may either be taken as evidence of an intermediate form or simply be produced by an induced CD onto the B-DNA spectrum due to the drug-base electronic interactions [17].

Furthermore, when half of the sample of poly[d(G-m 5 C)] plus mitomycin, in Buffer Z, used in the ^{31}P -n.m.r. studies shown in Figure 3(d), was dialysed extensively against Buffer B it rendered a c.d. spectrum consistent with a B-form of DNA (Figure 4b, line 1), but displayed a Z-like spectrum when the dialysis was made against Buffer Z (Figure 4b, line 2). Interestingly, if we confidently assume (for the reasons given above; see Table 1) that the drug

Figure 4 C.d. of sonicated poly[d(G-m⁵C)]-mitomycin complexes in the absence of unbound drug

(a) The sample displayed in Figure 3(b) dialysed against Buffer B (—) or Buffer Z (---). (b) The sample displayed in Figure 3(d) dialysed against Buffer B (—) or Buffer Z (---).

Figure 5 ³¹P-n.m.r. of a poly[d(G-m⁵C)]-mitomycin complex in the absence of unbound drug

This corresponds to the sample whose c.d. spectrum is shown in Figure 4(b), ----.

binds to the B-form, but not significantly to the Z-form, the spectra should represent a clear example of an intermediate in a B → Z transition, with the drug bound to B-form polynucleotide while other portions of the spectrum are in the Z conformation owing to the presence of 3 mM MgCl₂. The ³¹P-n.m.r. spectrum (Figure 5) resembles the control Z-form one, with hardly appreciable sharp signals at -0.33 and -1.53 p.p.m., in agreement with their relationship with the non-covalent bound mitomycin C. These results might suggest that mitomycin C can only cross-link a few guanine bases in B domains within the Z-form polymer.

DISCUSSION

In the present study we used c.d. and ³¹P-n.m.r. to characterize the structure of poly[d(G-m⁵C)] modified by reductively activated mitomycin C. C.d. spectra are widely used to measure the effect of bound drug on the B → Z transitions. In most cases the ligand appears to work as an inhibitor of the B → Z conformational change and as allosteric effector of the Z → B conformational

transition [12–15], favouring the B conformation. The cases of Z → B reversion described previously [12–15, and references cited therein] were obtained with drugs which bind reversibly to DNA, except in a particular and enlightening case in which ethidium was covalently bound to DNA [14]. At first glance, mitomycin C should have an advantage when used to measure such a transition in that the mitomycin bis-adduct is irreversible; thus we can be sure that the drug is, for example, only bound to fragments in the B-conformation, while other regions remain in the Z-conformation.

Mitomycin binding to B-form poly[d(G-m⁵C)] does not produce a transition to Z-form DNA, as unambiguously established by the ³¹P-n.m.r. experiments (Figure 3b), despite the characteristics of the c.d. spectra in Figure 2(a), and in clear agreement with the data obtained with other DNAs [5,17,18]. The results described here corroborate the view that covalently attached drugs might nucleate B-regions which remain irreversibly in this conformation. These drugs, and many intercalating agents which bind to DNA through the minor groove, appear to possess high effectiveness as inhibitors of the B → Z transition [12–15]. In contrast, it is remarkable that platinum anti-tumour drugs, which cross-link DNA through the major groove [29], behave in a different way, since they increase the Z-DNA content in plasmids with G + C-rich inserts [30].

The Z → B-form reversion in poly[d(G-C)] has been described in the presence of bis-adducts of mitomycin C. This reversion arises from the cross-linking of the drug to B-regions which might exist in the overall Z-polynucleotide [5] followed by stabilization of the B-form, producing the Z → B conformational change. The only explanation for the absence of a Z → B conformational reversion in the experiments using poly[d(G-m⁵C)] (Figures 2a, 3 and 5) is that the drug binds neither to the Z-form of the polymer nor 'sufficiently' to the B-zones within it, tentatively because the Z-form of this polymer is more stable as a Z-form polynucleotide under low salt conditions and behaves as a 'less-mobile' polymer (i.e., the cross-link produces greater rigidity in the modified polynucleotide). Therefore, poly[d(G-m⁵C)] is neither reverted to the B-form nor sufficiently cross-linked by the bifunctionally activated mitomycin under Z-forming conditions. Mitomycin C fits snugly into the minor groove of Z-DNA, but its C-1 and C-10 atoms (see Figure 1) are not as close to the N-2 atoms of the polynucleotide as they are in the B form [5–6], in which the minor groove can widen to about 0.1 nm (10 Å), facilitating the cross-link [6]. The binding ratios displayed in Table 1 corroborate the view that mitomycin only binds slightly to the small equilibrium fraction of B-form, but is unable to induce the Z → B transition in poly[d(G-m⁵C)]. Moreover, the binding ratios of mitomycin to the B-conformer of poly[d(G-m⁵C)] should be higher than for poly[d(G-C)] as is the case (cf. Table 1 and [21]) and in agreement with the preferred cross-linking of mitomycin C to methylated GpC tracts [21].

In summary, mitomycin C appears to inhibit the B → Z transition of poly[d(G-m⁵C)], even in the presence of 3 mM MgCl₂, while neither the Z-form of poly[d(G-m⁵C)] nor the B-like regions in the high-salt form of the polynucleotide interact significantly with the drug under bifunctionally activating conditions; thus no reversion from the Z-form to the B-form of the polynucleotide can be observed under salt conditions which are required for the Z-form to exist. As described in detail in the Introduction, studies on methylated regions in DNA emphasize the important role that B → Z transitions might play in the control of gene activity [9–11]. Studies like that presented here not only give a clue to the possible mechanism of action of the anti-tumour drug mitomycin C on guanine + cytosine regions in DNA, but also offer additional information about the effect of

small ligands binding on DNA, thereby providing us with new details about the nature and potential role of the Z and B conformations of DNA.

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