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Evaluation of the structural modifications induced by mitomycin C on nucleic acids

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Abstract

The interaction of poly(dG-dC) · poly(dG-dC) with mitomycin C, an antitumor antibiotic, has been studied by various spectroscopic methods: circular dichroism, Fourier transform infrared resonance Raman scattering and using fluorescence emission of terbium bound to unpaired guanines as local conformation probe. The results allowed us to confirm the lack of long range modification of the DNA secondary structure upon binding. They also brought first information concerning the modification of the local structure of the nucleic acid at the level of mono- or bifunctional adducts.

Keywords: Mitomycin C; DNA–drug interaction; Terbium as a conformational probe

1. Introduction

Mitomycin C (Fig. 1) is an effective antibiotic and an antitumor agent of clinical use. It reacts with DNA at the N_2 position of guanine. Whereas its effect is identical to that of other alkylating antitumor agents, mitomycin C is particular because it needs a reductive activation to react with DNA. *In vitro*, this reduction can be carried out by the use of NADPH/microsomal system, H_2/PtO_2 reductive system, dithionite [1,2] or a moderate acidic treatment [3]. The enzymatic or chemical reduction induces an opening of the aziridine ring of the molecule with the appearance of a reactive center at the C_1 position, which undergoes a monofunctional interaction with DNA. A second reactive center has been described at the C_{10} position [1,4,5] which explains the bifunctional activity of the drug.

It is possible, according to the reductive pathway of mitomycin C, to favour the formation of mono- or bifunctional adducts. The H_2/PtO_2 system favours the production of monoadducts, whereas dithionite ($Na_2S_2O_4$) more readily leads to crosslinks [6]. In the latter case, the ratio monoadducts/crosslinks seems to depend on the procedure: the fractionated addition of $Na_2S_2O_4$ favours crosslinks [7]. The crosslinks ratio is also enhanced by a pH decrease [8].

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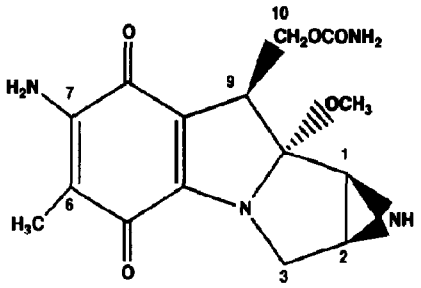

Fig. 1. Mitomycin C structure.

Whereas the monofunctional alkylations are 10 to 20 times more frequent than the bifunctional ones [1,9], the crosslinks have been considered for a long time as the cause of the lethal effect upon the cancerous cells [1]. Nevertheless, the amount of crosslinks is not different in sensitive or resistant cells to mitomycin C [10]. Moreover, it has been shown that the monofunctional alkylations can induce important damages [5,11–13]. Among these are single strand breaks induced by the hydroxyl radicals and hydrogen peroxide formed during the reoxidation of the hydroquinone [14,15]. It has recently been shown that a good correlation exists between the formation of free radicals and the cytotoxicity of the drug in sensitive cells [10].

In this paper, a set of spectroscopic methods allowed us to precise the conformational modifications of B-DNA after its alteration by mitomycin C. Since guanine is the only known target of the antibiotic and since there is a remarkable base-sequence specificity of dCpG not only at the level of crosslinks [7,8,16] but also at the level of the monoadducts [17], B-form of poly(dG-dC)·poly(dG-dC) was chosen as a model of bonding of mitomycin C on dCpG sequences. Some of the used methods supply information concerning the polynucleotide as a whole: these are circular dichroism (CD), Fourier transform infrared (FTIR) and UV resonance Raman spectroscopy (RRS). In contrast, terbium which binds to unpaired guanines has been used as a local conformation probe at the level of the adduct.

2. Materials and methods

Mitomycin C was supplied by Boehringer Mannheim and used without further purification. Poly(dG-dC)·poly(dG-dC) was supplied by Pharmacia Biochemical, sodium dithionite ($Na_2S_2O_4$) by Fluka, PtO_2 by Jansen and Terbium(III) chloride hexahydrate by Aldrich.

2.1 Preparation of mitomycin C–poly(dG-dC)·poly(dG-dC) complexes under various activating conditions

$Na_2S_2O_4$ activation was used for CD, FTIR and RRS experiments according to a modified procedure from Iyer and Szybalski [1] and Tomasz et al. [6,18]. The polynucleotide (1 to 2 μmol) and mitomycin C (excess of 2 to 3 mol:mol) were mixed in 15 m*M* Tris buffer, pH 7.4, and deaerated by bubbling helium gas through the solution for 20 min. The reducing agent $Na_2S_2O_4$ was freshly prepared by adding the solid into deaerated water and was added in 5 equal portions at 5 min intervals to a final excess of 2 mol $Na_2S_2O_4$ for 1 mol mitomycin C. Helium bubbling continued for 45 min then the mixture was exposed to air. As a result, the blue color of mitomycin C changed to dark purple.

Two different procedures were used for fluorescence experiments: (i) $Na_2S_2O_4$ activation: 0.11 μmol of poly(dG-dC)·poly(dG-dC), 0.34 μmol of mitomycin C and 0.68 μmol of $Na_2S_2O_4$ were mixed in a total volume of 1 ml containing 10 m*M* Tris buffer, pH 7.4. Deaeration was processed as described before. (ii) PtO_2/H_2 activation is a modified procedure from Tomasz [6]: 0.11 μmol of poly(dG-dC)·poly(dG-dC), 0.34 μmol of mitomycin C and 0.3 mg solid PtO_2 catalyst were mixed in a total volume of 1 ml containing 27 m*M* phosphate buffer, pH 7.4. The mixture was deaerated by helium bubbling, followed by a 5 min reduction by hydrogen bubbling. Helium was then again bubbled for 5 min, reaeration was carried out and the same color change as before appeared. PtO_2 was eliminated by centrifugation.

Whatever the procedure, the polynucleotide–mitomycin C complex was separated from the

unbound antibiotic by chromatography on a G-100 or a PD-10 Pharmacia column equilibrated with 10 m*M* Tris buffer, pH 7.4.

2.2 *Spectroscopic methods*

Circular dichroism (CD) spectra were recorded on a Jouan-Roussel IV dichrograph connected to a microcomputer. FTIR spectra were recorded using a Perkin–Elmer 1760 spectrophotometer. The spectral resolution was 1 cm^{-1} and the spectra were accumulated 10 times. Spectra were obtained in H_2O or D_2O solutions and in hydrated films. Data treatement performed on a Perkin–Elmer 7700 computer included baseline correction, normalization and smoothing using the Savitsky and Golay algorithm. Resonance Raman spectra with a 257 nm excitation wavelength have been obtained by the use of the microRaman spectrophotometer already described [19]. All the spectra were normalized. The concentrations of reference and modified poly(dG-dC)·poly(dG-dC) were the same and the 1340 cm^{-1} water bond was used as internal standard. The Raman spectrum of mitomycin C metabolites was normalized in such a way it allows the best fit when reconstructing by the use of a multilinear regression method the spectrum of the modified polynucleotide from those of the unmodified poly(dG-dC)·poly(dG-dC) and of the metabolites.

The method used for terbium fluorescence measurements has already been described [20]. $TbCl_3 \cdot 6H_2O$ was added to control or modified poly(dG-dC)·poly(dG-dC) at a final concentration equal to four times the nucleotide concentration. Fluorescence was measured on a drop by using the microspectrofluorometer already described [21]. The excitation wavelength was 290 nm [20,22] and the fluorescence spectra were recorded between 390 nm and 620 nm.

3. Results and discussion

A number of structural changes of DNA induced by mitomycin C have already been described, either at the secondary structure level or about the flexibility of the overall molecule [23].

Tomasz and collaborators [24] first measured the modifications of the CD spectrum of poly(dG-dC)·poly(dG-dC) after binding of mitomycin C. At high binding ratio of ligand (0.25), it becomes strikingly similar to the inverted spectrum observed with the Z-DNA [25]. These inversions of the spectrum have also been observed for complexes of mitomycin C with calf thymus, *E. coli*, T_2 phage DNA [24] or *Micrococcus lysodeikticus* DNA [26].

However, ^{31}P NMR and radioimmunoassay studies [26] have ruled out Z-DNA conformation in the mitomycin C–polynucleotide complexes. Tomasz et al. [26] also prooved that mitomycin C inhibited the conversion by ethanol of poly(dG-dC)·poly(dG-dC) to the Z-form and more recently Rao and collaborators [27] have presented a molecular mechanic simulation showing that mitomycin C locks bases in the *syn* conformation and then prevents the B → Z conformation transition of the DNA.

The modifications of the CD spectrum are then attributed to a drug-induced left-handed but non-Z conformational change or to the superposition of an induced CD onto the CD of B-DNA due to drug–base electronic interactions [26].

Using the same procedure (see Section 2) which favours the appearance of crosslinks,we checked the possibility of a global modification of the structure of poly(dG-dC)·poly(dG-dC) after its reaction with mitomycin C by the use of FTIR and resonance Raman spectroscopies.

Figure 2 presents the FTIR spectra of poly(dG-dC)·poly(dG-dC) in its B (Fig. 2b) and Z-forms (Fig. 2a) as well as the spectrum of poly(dG-dC)·poly(dG-dC) modified by mitomycin C (Fig. 2c). The IR absorption bands characteristic of the B and Z conformations of poly(dG-dC)·poly(dG-dC) have been dotted (B-form) and hatched (Z-form). A complete set of assignments has been given previously [28]. None of the Z-form marker bands is detected on the FTIR spectrum of the poly(dG-dC)·poly(dG-dC)-mitomycin C complex (Fig. 2c), confirming that the drug does not induce the left-handed conformation of the polynucleotide.

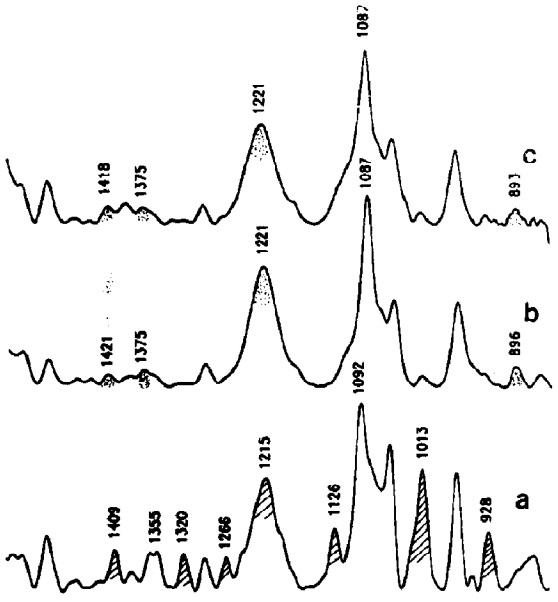

Fig. 2. FTIR spectra of Z-form (a) and B-form (b) of poly(dG-dC)·poly(dG-dC) and of poly(dG-dC)·poly(dG-dC) modified by mitomycin C (c).

In the same conditions of modification, CD spectra of reference and modified poly(dG-dC)·poly(dG-dC) are presented on Fig. 3. We recognize, in the case of the modified polynucleotide, the characteristics looking like those of the left-handed poly(dG-dC)·poly(dG-dC): positive peak near 260 nm, decrease of the CD above 280 nm.

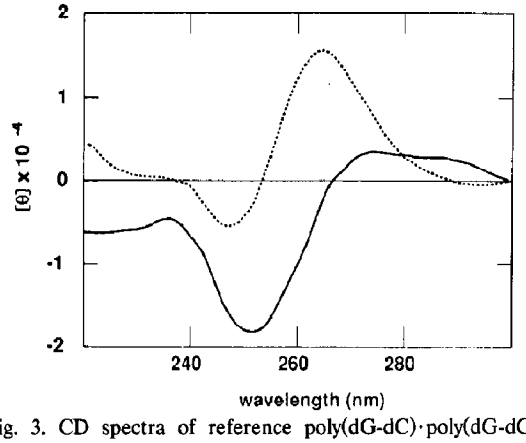

Fig. 3. CD spectra of reference poly(dG-dC)·poly(dG-dC) (solid line) and of poly(dG-dC)·poly(dG-dC) modified by mitomycin C (dotted line).

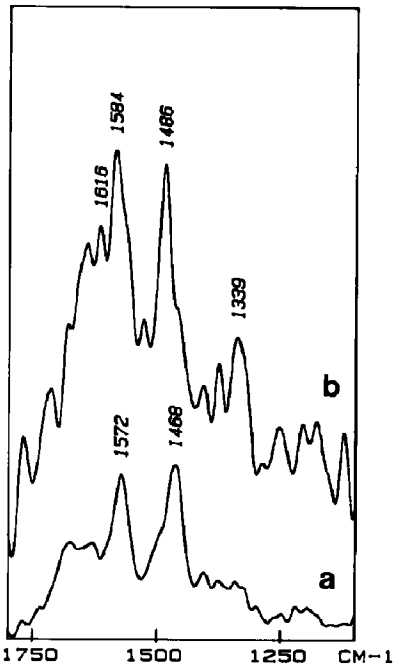

Fig. 4. Resonance Raman spectra of mitomycin C metabolites (a) and of poly(dG-dC)·poly(dG-dC) modified by mitomycin C (b). $\lambda_{exc} = 257$ nm.

The FTIR spectra of the complex and B-form of poly(dG-dC)·poly(dG-dC) are almost exactly superimposable; all the characteristic lines of the B-form of poly(dG-dC)·poly(dG-dC) are present. We can thus assume that there is no modification of the secondary structure of the nucleic acid: its global conformation is still a B-form.

In resonance Raman spectroscopy only chomophores susceptible to absorb the excitation wavelength contribute to the spectrum. Therefore with a 257 nm excitation wavelength, both poly(dG-dC)·poly(dG-dC) and mitomycin C contribute to the resonance Raman spectra since their respective molar absorption coefficients are about 8500 and 14000 M^{-1} cm^{-1} respectively. The mitomycin C metabolites were obtained by the $Na_2S_2O_4$ reductive system. Their vibrational contribution (Fig. 4a) to the resonance Raman spectrum of mitomycin C modified poly(dG-dC)·poly(dG-dC) (Fig. 4b) appears as a shoulder in the 1486 cm^{-1} and 1584 cm^{-1} lines. Figure 5 presents the spectra of reference poly(dG-dC)·poly(dG-dC) (Fig. 5a) and poly(dG-dC)·poly(dG-

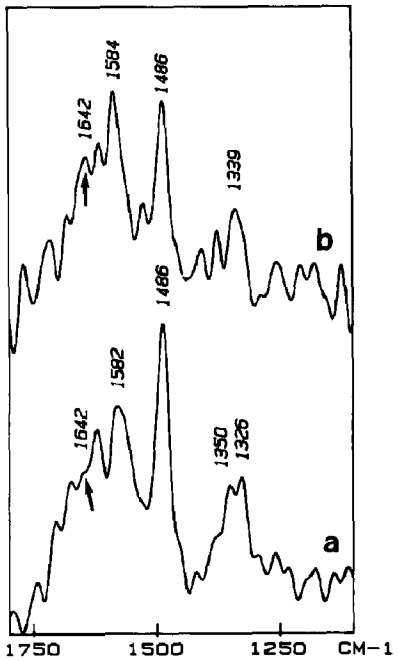

Fig. 5. Resonance Raman spectra of reference poly(dG-dC)·poly(dG-dC) (a) and of poly(dG-dC)·poly(dG-dC) modified by mitomycin C minus the contribution of mitomycin C metabolites (b). $\lambda_{exc} = 257$ nm.

dC) modified by mitomycin C after subtraction of the contribution of mitomycin C metabolites (Fig. 5b; see Section 2).

The main differences in the spectra presented in Figs. 5a and 5b are the increase of a 1642 cm^{-1} line (arrows) and the decrease of the 1486 cm^{-1} line for the modified polynucleotide. The ratio intensity decrease of the lines located at 1486 cm^{-1} and 1584 cm^{-1} is the result of the chemical modification at the N_2 position of guanine [29]. The 1642 cm^{-1} line is the only visible contribution of cytosine to the resonance Raman spectrum of poly(dG-dC)·poly(dG-dC) [30] at this excitation wavelength. It has been assigned to the cytosine $_2C{=}O$ stretching motion [31]. This cytosine line appears only in the double-stranded polynucleotide spectra and its increase has been correlated to a more important interbase vibrational coupling of the carbonyls [30,32]. This increase of coupling could be correlated to an enhancement of the rigidity of the macromolecule which is consistent with the interstrand crosslinks favoured by the dithionite reduction. These crosslinks stabilize the double helix structure of poly(dG-dC)·poly(dG-dC) and restrict its dynamic breathing motions. The same considerations could explain the modifications of the rheodynamic properties (increased viscosity) observed by Kaplan and Tomasz [23] in the case of the nucleic acids modified by mitomycin C.

Terbium is a sensitive probe of guanine bases present not only in single-stranded denatured segments of DNA [33] but also in local, non-denatured distortions of the double helical DNA, in which hydrogen bonds can be preserved but vertical stacking of base-pairs is altered [20]. Gross and Simpkins [22] have proposed that the N_7 and C_6O groups of guanine are coordinately bound by terbium, which leads to a great enhancement of the intrinsic fluorescence of terbium by intramolecular energy transfer from DNA-mitomycin C complexes to terbium. Since guanines are the only target of mitomycin C, terbium can be considered as a chemical probe of local conformational modification after alteration of nucleic acids by mitomycin C.

Poly(dG-dC)·poly(dG-dC) was modified by mitomycin C through two reductive pathways favouring respectively the formation of crosslinks (use of dithionite) and the formation of monoadducts (use of the PtO_2/H_2 system) (see Section 2).

The fluorescence emission results obtained after reaction of Tb^{3+} are presented on Fig. 6. With a 290 nm excitation wavelength, we observe in the 390 nm–620 nm range, a main band around 540 nm and three smaller bands at 490 nm, 583 nm and 616 nm (not shown). The Tb^{3+} fluorescence would be 40 times more intense with poly rG (guanine integrated in a rather disordered structure) than with poly(dG-dC)·poly(dG-dC) (not shown).

It appears that when reduction is carried out with dithionite (which favours crosslinks), the fluorescence intensity of Tb^{3+} (Fig. 6b) is the same or probably lower than in reference poly(dG-dC)·poly(dG-dC) (Fig. 6a). On the opposite, when reduction is carried out with PtO_2/H_2 (which favours monoadducts), the fluorescence intensity

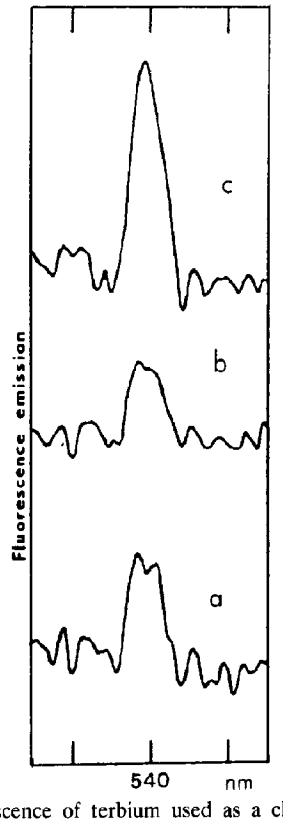

Fig. 6. Fluorescence of terbium used as a chemical probe: reference poly(dG-dG) · poly(dG-dC) (a), poly(dG-dC) · poly(dG-dC) modified by mitomycin C by the use of $Na_2S_2O_4$ (b), poly(dG-dC) · poly(dG-dC) modified by mitomycin C by the use of PtO_2/H_2 (c). $\lambda_{exc} = 290$ nm.

of Tb^{3+} (Fig. 6c) is about twice as important as in reference poly(dG-dC) · poly(dG-dC).

These results indicate (i): a local deformation at the level of the monofunctional adducts and consequently a greater flexibility of the chain around the monofunctional adducts, (ii): a greater rigidity around the crosslinks, which confirms our resonance Raman spectroscopic results and is in good agreement with the interpretation of rheodynamic measurements [23].

In conclusion, we have shown, by means of spectroscopic studies, that binding of mitomycin C on polynucleotides did not induce modifications of the secondary structure of the double helix. However, from preliminary studies in which terbium was used as local deformation probe, it appears which local deformations have long-range effects and result in modification of intrinsic properties of DNA, such as flexibility or breathing motions.

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