FEBS Letters 372 (1995) 144–147 FEBS 16040

Analogue versus digital recognition of DNA by bleomycin: an effect of the carbohydrate moiety

Christian Bailly[a], Abderraouf Kénani[a,**], Michael J. Waring[b,*]

[a]*Institut de Recherches sur le Cancer, INSERM U124, Place de Verdun, 59045 Lille, France*
[b]*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK*

Received 31 July 1995

Abstract **We have sought to determine the influence of the carbohydrate moiety of the antitumour antibiotic bleomycin on the sequence-specific cleavage of DNA. Both bleomycin A_2 and deglycobleomycin A_2 produce different cleavage patterns with DNA in which the 2-amino group has been removed from guanine, added to adenine, or both, as well as on a designed DNA fragment containing a few defined cleavage sites. Although each drug cleaves DNA primarily at GpT and GpC sites, the cleavage at these sites is frequently found to be stronger with deglycobleomycin compared with bleomycin A_2. Conversely, in most cases the cleavage at secondary sites, in particular at ApT steps, is significantly reduced or even abolished with deglycobleomycin. The results indicate that the gulose-mannose moiety of bleomycin A_2 plays a significant role in the recognition of preferred nucleotide sequences and confirm the view that both secondary structure and interaction with guanine are involved in determining sequence-specific cleavage of DNA by bleomycin.**

Key words: Bleomycin; DNA recognition; DNA cleavage; Sequence specificity

1. Introduction

The bleomycins, used in cancer chemotherapy for more than 20 years, cleave nucleic acids sequence-specifically in the presence of oxygen and certain metal ions [1,2]. The structure of bleomycin A_2 (Fig. 1) — the major component of the clinical formulation — is commonly divided into three functional domains: (i) the bithiazole and sulphonium side-chain which provide a DNA-binding element [1–3], (ii) the pseudopeptide portion which acts as a metal chelating/oxygen activation domain [1,2] and (iii) the carbohydrate moiety which may help to complex the metal, stabilise the reactive oxygenated species [4–6] and facilitate penetration into cells [7]. However, the exact role of the gulose-mannose disaccharide remains uncertain.

Although the bithiazole contributes significantly to the interaction with DNA, different studies suggest that it is the pseudopeptide moiety which is mainly responsible for sequence recognition [8–10]. Two recent models based on interaction with oligonucleotides [11,12] differ as regards the mode of binding of the bithiazole unit but both confirm that the pseudopeptide constitutes a prime element for sequence-specific binding to DNA. In addition, NMR studies suggest that the carbohydrate portion could contribute to the interaction since intermolecular NOEs between the gulose and the central thymidine in the sequence $d(CGCTAGCG)_2$ have been detected [12]. We have explored this idea by comparing the patterns of cleavage by bleomycin A_2 and deglycobleomycin A_2. We report that the sugar moiety of bleomycin is not mandatory for cutting at primary GpT and GpC sites but it plays a decisive role in cutting at secondary sites, particularly ApT.

*Corresponding author. Fax: (44) (1223) 334 040.
E-mail: mjw11@cus.cam.ac.uk

**Present address:* Faculty of Medicine, 5019 Monastir, Tunisia.

Abbreviations: bp, base pair(s).

2. Materials and methods

2.1. Bleomycin A_2 and deglycobleomycin

A pure sample of bleomycin A_2 was obtained from Roger-Bellon Laboratories (Neuilly-sur-Seine, France). The gulose-mannose moiety of bleomycin A_2 was specifically cleaved by HF solvolysis according to a published protocol [13]. Deglycobleomycin A_2 was purified by HPLC and the integrity of the deglycosylated antibiotic was checked by [^{1}H]NMR and HR-FAB mass spectrometry. Stock solutions were prepared in 10 mM Tris-HCl buffer containing 10 mM NaCl, pH 7.0, divided into 250-μM aliquots and stored at −20°C.

2.2. DNA fragments

The 48-bp AT-rich fragment was cloned between the *Hin*dIII and *Bam*HI sites of the pUC12 vector to afford the plasmid construct pCB2 [14]. Four additional restriction fragments were used to investigate cleavage of DNA by bleomycin and deglycobleomycin: (i) a 178-bp *Eco*RI-*Pvu*II fragment from pUC12, (ii) a 117-bp fragment and (iii) a 265-bp fragment both cut out of plasmid pBS with *Eco*RI and *Pvu*II, and (iv) the 160-bp *tyr*T(A93) fragment cut out of plasmid pKMp27. The four different 160-bp DNA fragments containing either natural bases or inosine residues in place of guanines or 2,6-diaminopurine residues (abbreviated D within a sequence for clarity) in place of adenines or both I and DAP residues were synthesized by PCR amplification as described recently [15,16]. Restriction fragments were 3′-end-labelled with [α-^{32}P]dATP in the presence of avian myeloblastosis virus reverse transcriptase. The purified PCR products were 5′-end-labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase according to a standard procedure for labelling blunt-ended DNA fragments. After completion of the labelling reaction, the DNA was again purified by electrophoresis on a 6% polyacrylamide gel. Finally, the radioactive DNA was resuspended in 10 mM Tris-HCl, pH 7.0, buffer containing 10 mM NaCl.

2.3. Cleavage of DNA by the bleomycin- and deglycobleomycin-Fe^{II} complexes

In a typical experiment, the freshly prepared bleomycin–Fe^{II} complex (4 μl) was added to 6 μl of [^{32}P]-end-labelled DNA (~1 nM) in 10 mM Tris-HCl buffer, pH 7.0, containing 10 mM NaCl. The bleomycin–Fe^{II} complex consisted of 2 μl of a 5-μM solution of bleomycin A_2 mixed with 2 μl of 5 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ just prior to the experiment. After 1 min incubation at room temperature, the cleavage reaction was stopped by freezing. Samples were lyophilized, resuspended in 50 μl of water and lyophilized again. With deglycobleomycin, the digestion time was 2 min to compensate for the slightly lower efficiency of DNA cleavage by the deglycobleomycin–Fe^{II} complex compared with the bleomycin–Fe^{II} complex. The cleavage products were resuspended in

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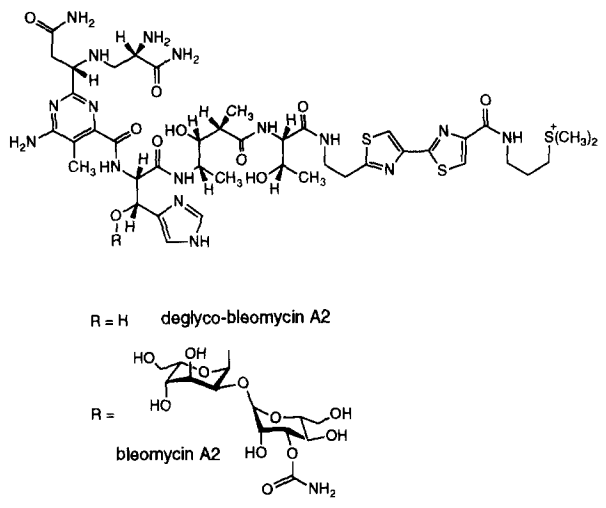

Fig. 1. Structures of bleomycin A_2 and deglycobleomycin A_2.

1 μl of formamide-dye solution and resolved on a denaturing polyacrylamide gel as described below.

2.4. Electrophoresis and quantitation by storage phosphor-imaging

DNA-cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After the electrophoresis (~2.5 h at 60 W, 1600 V in TBE buffer, BRL sequencer model S2), gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80°C and examined by autoradiography using either a phosphorimager or X-ray films (Fuji R-X) exposed at −70°C with an intensifying screen usually for 24 h. For quantitative analysis, a Molecular Dynamics 425E PhosphorImager was used to collect data from storage screens exposed to the dried gels overnight at room temperature. Base line-corrected scans were analysed by integrating all the densities between two selected boundaries using ImageQuant Version 3.3 software. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards (G or G + A tracks).

3. Results and discussion

Recently, we showed that the 2-amino group of guanine directs sequence-specific cleavage of DNA by bleomycin [17]. Cutting of DNA by deglycobleomycin also depends on the location of that group. Fig. 2 shows comparative cleavage patterns on a series of homologous *tyr*T(A93) DNA molecules containing inosine and/or 2,6-diaminopurine residues in place of guanosine and/or adenine residues, respectively. With both bleomycin and its deglycosylated analogue, the cleavage pattern varies considerably depending whether the purine 2-amino group is present on guanine, transferred to adenine, present on both or missing altogether. Furthermore, the intensity of strand scission at a given bond frequently differs for the two drugs: a site of strong cleavage with bleomycin sometimes corresponds to a weak site for deglycobleomycin or vice versa (Fig. 4). The chief outcome is that with normal DNA most, but not all, GpC and GpT steps (the reactive nucleotide is underlined) appear

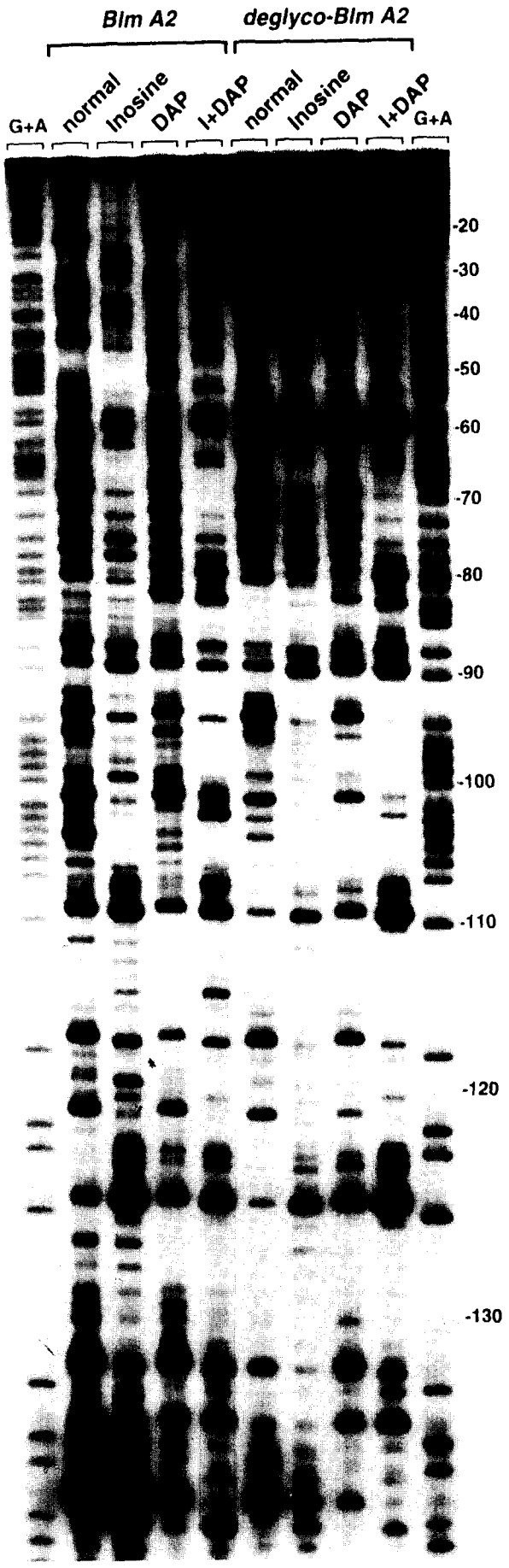

Fig. 2. Cleavage by the Fe^{II} complex of bleomycin A_2 and deglycobleomycin (1 μM each) of 5′-end-labelled *tyr*T(A93) DNA species containing natural or modified nucleotides.

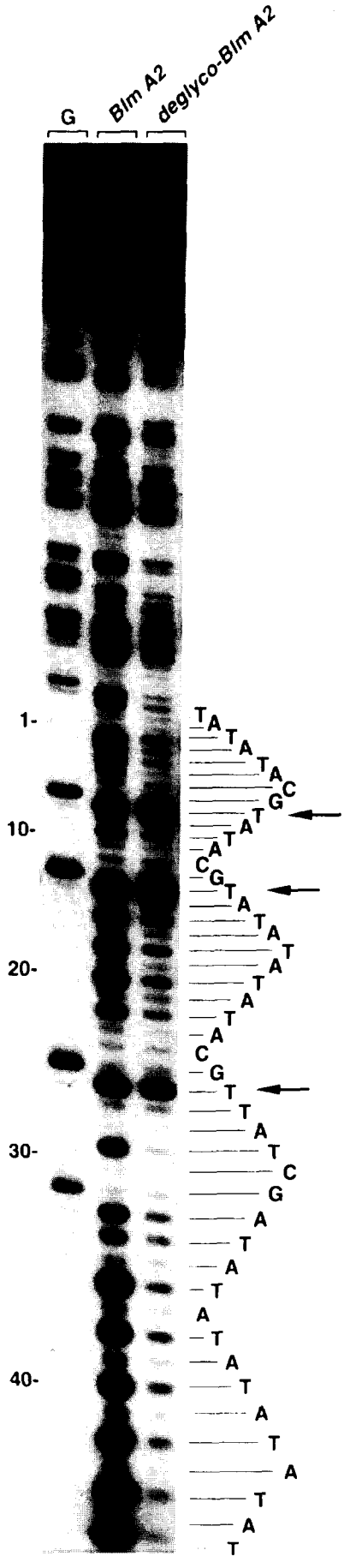

Fig. 3. Cleavage by the Fe^{II} complex of bleomycin A_2 and deglycobleomycin (1 μM each) of a 170-mer DNA fragment containing the indicated insert.

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more susceptible to cleavage by deglycobleomycin compared with bleomycin whereas cleavage at secondary sites is frequently reduced with the deglycosylated derivative (e.g. ApT 109 and GpA 104). The results with the doubly substituted I + DAP DNA are totally consistent with this conclusion since cleavage at DpC and DpT sites is stronger with deglycobleomycin than with bleomycin A_2 (steps 59, 89, 109 and 125) whereas cleavage at IpC or IpT remains weak or is even abolished (steps 94, 117 and 137).

We confirmed this observation using other DNA fragments, including a designed sequence containing only three GpT sites flanked by $(A \cdot T)_n$ repeats. As shown in Figs. 3 and 4, bleomycin cuts efficiently at the GpT steps as well as within the adjacent $(A \cdot T)_n$ tract [18] whereas deglycobleomycin cuts almost exclusively at GpT. Altogether, these experiments establish that removal of the carbohydrate moiety of bleomycin results in stronger cleavage at GpPyr sites accompanied by diminished cutting at ApPyr sequences.

Experiments with the singly inosine-substituted DNA show that the 2-amino group of guanine is not mandatory for cleavage by either bleomycin or deglycobleomycin: strong cutting is still observed despite all purines lacking a 2-amino group, suggesting that the sequence recognition by both agents depends as much on the secondary structure as on the primary sequence per se. Indeed, unlike what is observed with normal DNA, strong cleavage can be detected at certain ApT sites in I-DNA (e.g. positions 89 and 109) although A · T bp have not been altered in this polynucleotide. With the DAP-DNA, as expected, cleavage can occur at both GpPyr and DpPyr steps revealing that the interaction of either drug with the purine 2-amino group is sufficient (but not necessary) to create new cleavage sites.

Conclusion: Evidently, the carbohydrate moiety of bleomycin affects its recognition of particular nucleotide sequences. More specifically, the fact that bleomycin and its deglycosylated analogue respond differently to the location of the purine 2-amino group in DNA raises the possibility that the gulose-mannose portion of the antibiotic influences recognition via the minor groove width. The transfer of the 2-amino group from guanine to adenine residues significantly modulates the width of the minor groove in DNA as judged from experiments with the conformation-sensitive probes DNase I and uranyl nitrate [16]. Our results reinforce the view that sequence-specific cleavage of DNA by bleomycin is the result of a direct recognition of the guanine 2-amino group coupled with indirect recognition of a suitable minor groove width [1,12,17]. Whereas the carbohydrate domain of bleomycin apparently contributes little to primary sequence recognition (digital read-out), it seems to contribute noticeably to the DNA-structure recognition (analogue read-out). In this regard, bleomycin behaves like other antibiotics equipped with carbohydrate substituents, such as calicheamicin and chromomycin [19].

Acknowledgments: We thank D. Gentle for his invaluable technical assistance. This work is a contribution from the Cambridge Centre for

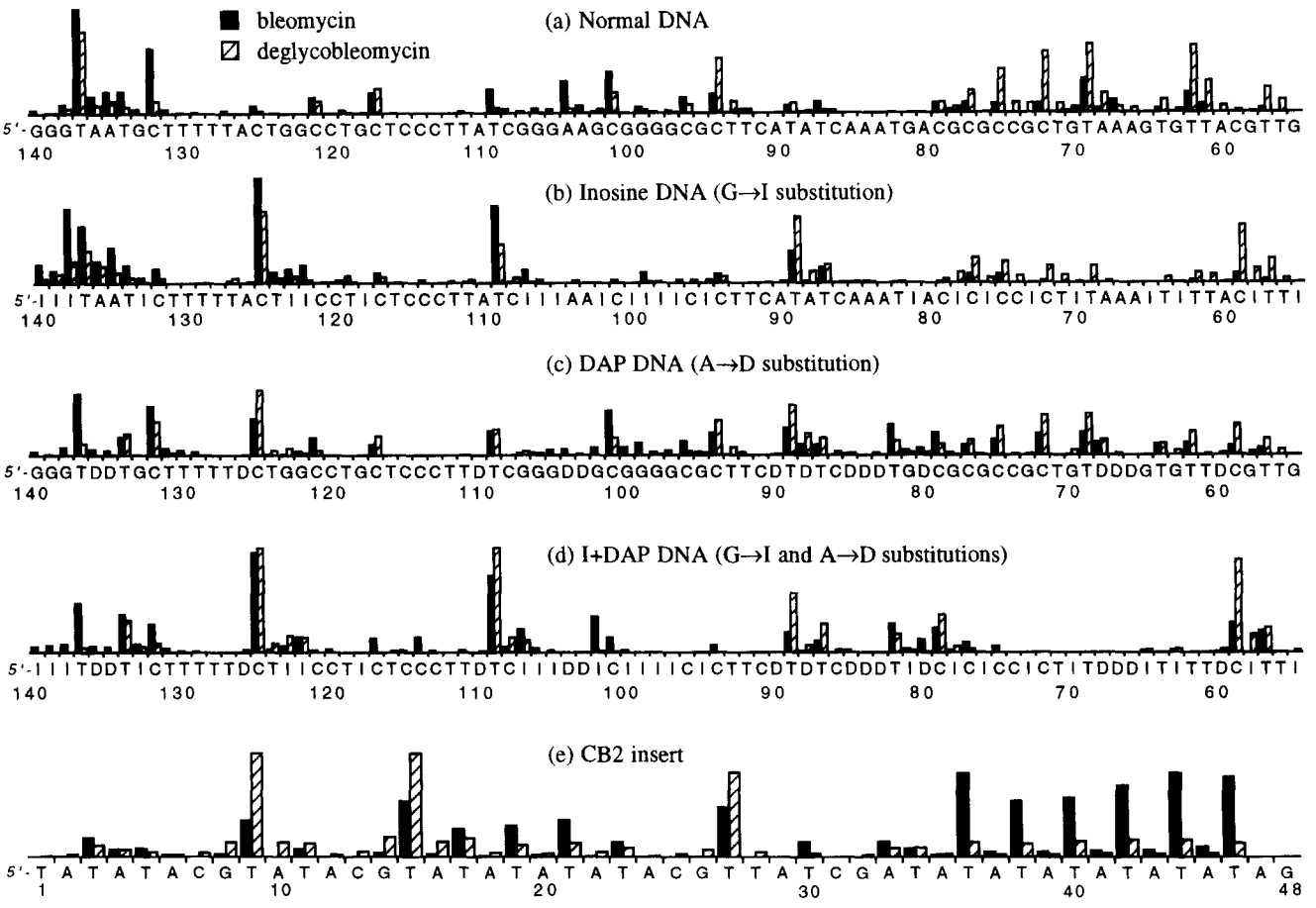

Fig. 4. Comparison of the susceptibility of normal and modified *tyr*T(A93) DNA (a–d) and the AT-rich insert sequence CB2 (e) to cleavage by bleomycin and deglycobleomycin. The relative cleavage intensity at a given bond was measured as a fraction of the total cleavage of all the phosphodiester bonds within the sequence.

Molecular Recognition and was supported by grants (to M.J. Waring) from the Cancer Research Campaign, the Wellcome Trust and the Association for International Cancer Research. C. Bailly thanks the ARC and the Sir Halley Stewart Trust.

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