

The DNA sequence specificity of bleomycin cleavage in telomeric sequences in human cells

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Abstract Bleomycin is an antibiotic drug that is widely used in cancer chemotherapy. Telomeres are located at the ends of chromosomes and comprise the tandemly repeated DNA sequence $(GGGTTA)_n$ in humans. Since bleomycin cleaves DNA at 5'-GT dinucleotide sequences, telomeres are expected to be a major target for bleomycin cleavage. In this work, we determined the DNA sequence specificity of bleomycin cleavage in telomeric sequences in human cells. This was accomplished using a linear amplification procedure, a fluorescently labelled oligonucleotide primer and capillary gel electrophoresis with laser-induced fluorescence detection. This represents the first occasion that the DNA sequence specificity of bleomycin cleavage in telomeric DNA sequences in human cells has been reported. The bleomycin DNA sequence selectivity was mainly at 5'-GT dinucleotides, with lesser amounts at 5'-GG dinucleotides. The cellular bleomycin telomeric DNA damage was also compared with bleomycin telomeric damage in purified human genomic DNA and was found to be very similar. The implications of these results for the understanding of bleomycin's mechanism of action in human cells are discussed.

Keywords Anticancer drug · DNA-damaging agent · DNA sequence specificity · HeLa cells and human cells · Telomere

Abbreviations

CGE-LIF Capillary gel electrophoresis with laser-induced fluorescence detection

ddATP Dideoxyadenosine triphosphate
ddCTP Dideoxycytidine triphosphate

Introduction

Bleomycin was first isolated from *Streptomyces verticillilis* and consists of a closely related family of glycopeptides [1, 2]. The interest in bleomycin lies not only in its antibiotic activity but also in its activity as a cancer chemotherapeutic agent, where it is used in the treatment of squamous cell carcinomas, germ cell tumours and certain types of lymphoma [3–6]. Bleomycin cleaves DNA to give both single-strand and double-strand breaks in vitro and in vivo [7], and this property is generally thought to be the major source of its cytotoxicity.

Bleomycin is composed of four functional domains: a metal-binding region, a linker region, a disaccharide and a bithiazole tail [8]. The metal-binding domain provides the coordination sites for complex formation with the transition metals Fe(II) and Cu(I) and molecular oxygen activation, which is responsible for the DNA cleavage [9]. The linker region not only provides a link between the metal-binding region and the bithiazole tail, but is also important for the efficiency of bleomycin binding to DNA [10]. The bithiazole tail assists in the affinity of bleomycin for DNA and may contribute to polynucleotide recognition and DNA cleavage selectivity [10, 11]. The disaccharide moiety is thought to be involved in bleomycin–cell recognition, cellular uptake of bleomycin and metal ion coordination [9].

When bleomycin in a metal-free form is administered intravenously into the bloodstream, it rapidly binds to Cu(II) in the blood serum to create a stable complex, bleomycin–Cu(II). When the complex is transferred inside a

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cell, bleomycin can be reduced to bleomycin–Cu(I). The new complex can enter the nucleus and form bleomycin–Fe(II) by exchange with Fe(II). This latter complex generates the “activated bleomycin” complex, bleomycin–Fe(III)–O–OH [12, 13]. The interaction of bleomycin with DNA involves the binding of the bithiazole ring to DNA through electrostatic interactions [14]. Bleomycin induces DNA damage in several ways, including strand scission of the DNA, and elimination of pyrimidine and purine bases from the DNA. At low concentrations of bleomycin, single-strand breaks are observed, whereas double-strand breaks occur at higher concentrations [15]. Bleomycin cleavage of the phosphodiester backbone of DNA mainly gives rise to 3′-phosphoglycolate and 5′-phosphate termini [16, 17].

Telomeres are G-rich tandem repeats that are located at the ends of chromosomes and the DNA sequence is (GGGTTA)_n in humans [18]. The average length of a human telomere is 8–20 kb [19–21]. At the end of the telomere, a single-stranded G-rich overhang, 50–300 nucleotides in length, invades the double-stranded telomere region to form a telomere loop, or T-loop. At the base of the T-loop, the invading strand displaces the identical strand of the duplex telomeric DNA to form the displacement loop, or D-loop. The 3′ end is shielded in the T-loop to protect it from DNA repair and other degradation activities [22, 23]. The T-loop is maintained by telomere binding proteins known as the shelterin complex [24–27].

The sequence specificity of bleomycin telomeric DNA damage in intact human cells was determined with a linear amplification polymerase stop assay [28, 29]. In this assay, *Taq* DNA polymerase extends from a fluorescently labelled oligonucleotide primer up to termination at the bleomycin cleavage site. The precise sites of bleomycin cleavage can be determined by comparison with dideoxy sequencing reactions using the same fluorescently labelled oligonucleotide primer and template. For the telomeric DNA sequence, a primer containing the telomeric repeat was used. The use of fluorescently labelled DNA fragments allowed capillary gel electrophoresis with laser-induced fluorescence (CGE–LIF) detection to be used as a method of analysis [30–34]. This recent method can separate DNA fragments at base-pair resolution with great accuracy and precision [32].

CGE–LIF detection has been used to investigate the DNA sequence specificity of DNA damage caused by cisplatin, cisplatin analogues and bleomycin in plasmid clones containing human telomeric DNA sequences [30, 32–34]. In a previous study, we examined the bleomycin DNA sequence specificity in a plasmid clone containing 17 tandem telomeric repeats [33].

We wished to extend our study with purified plasmid DNA to telomeric DNA sequences in human cells, since the sequence-specific interaction of bleomycin with cellular

telomeric DNA sequences has not been investigated previously. Bleomycin cleaves DNA preferentially at the sites adjacent to G nucleotides, such as GT, GA, GC and GG [35–37]. The human telomeric sequence (GGGTTA)_n contains the dinucleotides 5′-GT and 5′-GG, and thus it is expected to be a strong target sequence for the drug. Hence, the main objective of this project was to examine the telomeric DNA sequence specificity of bleomycin cleavage in human HeLa cells. To achieve this we used the linear amplification procedure with CGE–LIF detection as our method of analysis. The cellular bleomycin telomeric DNA damage was also compared with bleomycin telomeric damage in purified human genomic DNA. Data collected from this study will help improve our understanding of the antitumour mechanism of this drug.

Materials and methods

Bleomycin was purchased from Bristol Laboratories (USA) as the clinical preparation Blenoxane, which contains approximately 70 % bleomycin A₂ and 30 % bleomycin B₂. Bleomycin was dissolved in Milli-Q water to give a working stock solution of 10 mM. The high-performance liquid chromatography purified FAM-T21 oligonucleotide (5′-FAM-CCCTAACCCCTAACCC-3′) was obtained from Invitrogen.

For assessment of bleomycin damage of purified HeLa DNA, in a total volume of 20 μl, there was 12 μg of purified HeLa genomic DNA and equal concentrations of FeSO₄ and bleomycin (0, 0.03, 0.1, 0.2, 0.5, 0.7 and 1 mM). The reaction mixture was incubated at 37 °C for 30 min, followed by ethanol precipitation, and the precipitate was then redissolved in 20 μl of 10 mM tris(hydroxymethyl)amino-methane (Tris)–HCl, pH 8.8, 0.1 mM EDTA [38].

The HeLa cells were kindly donated by Noel Whitaker (University of New South Wales) and were grown in RPMI medium containing 10 % (v/v) fetal calf serum. After the cells had been washed twice with phosphate-buffered saline, 0.4 × 10⁵ cells were treated with 0, 0.1, 0.5 and 1.0 mM bleomycin in a total volume of 20 μl at 37 °C for 30 min [38–41]. After they had been washed with phosphate-buffered saline, the cells were incubated in 300 μl of 50 mM Tris–HCl (pH 7.5), 20 mM EDTA, with the addition of 6 μl of 10 % (w/v) sodium dodecyl sulfate and 3 μl of 10 mg/ml proteinase K, at 55 °C for 1 h, followed by phenol extraction and ethanol precipitation, and then they were redissolved in 20 μl of 10 mM Tris–HCl, pH 8.8, 0.1 mM EDTA. Then 1 μl of 2 mg/ml pancreatic RNase was added and the mixture was incubated at 37 °C for 30 min to degrade any remaining RNA [38]. The bleomycin treatment was monitored by agarose gel electrophoresis to gauge the extent of bleomycin DNA cleavage.

The oligonucleotide FAM-T21 was used as a primer to determine the bleomycin damage sites in the human telomeric DNA sequence. In a 20- μ l reaction, 5 μ g of DNA extracted from bleomycin-treated cells or 5 μ g bleomycin-treated purified HeLa DNA was combined with 10 pmol of FAM-T21, and the mixture contained 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl_2 , 25 μ M deoxynucleoside triphosphates (dNTPs), and 1 μ l of *Taq* DNA polymerase (note that the low concentration of dNTPs is required to observe the damage peaks). The reaction was thermally cycled in a Bio-Rad DNA Engine Dyad Peltier thermal cycler with the following parameters: 95 °C for 5 min and 35 cycles of 95 °C for 45 s, 57 °C for 1 min, 72 °C for 45 s and a final extension of 72 °C for 5 min.

The chain-terminating dideoxycytidine triphosphate and dideoxyadenosine triphosphate were used to create the sequence reference DNA size markers that allowed accurate determination of the bleomycin damage sites. In a 20- μ l reaction, 2 mM dideoxycytidine triphosphate or dideoxyadenosine triphosphate was combined with 10 pmol of FAM-T21, 25 μ M dNTPs, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl_2 , 5 μ g of purified human DNA and 1 U of *Taq* DNA polymerase. This was thermally cycled using the same parameters as above.

After linear amplification, the samples were ethanol-precipitated to remove artefact bands [34] and were redissolved in 5 μ l of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.8. A 2- μ l aliquot was analysed using an ABI 3730 capillary sequencer (Ramaciotti Centre for Gene Function Analysis, University of New South Wales) [33, 34]. Data from the ABI 3730 capillary sequencer were analysed with GeneMapper (Applied Biosystems) using a modified AFLP algorithm [34]. The experiments were repeated on at least three separate occasions and gave very similar results.

Results

HeLa cells were treated with various concentrations of bleomycin (0.1, 0.5 and 1.0 mM) and the DNA was extracted. This bleomycin-cleaved DNA was then subjected to linear amplification with the fluorescently labelled telomeric oligonucleotide FAM-T21 as a primer and analysed with CGE-LIF detection and GeneMapper. The results are presented as electropherograms, which show the damage position and the intensity of damage peaks (Figs. 1, 2). The no-drug control blanks showed a low level of DNA damage intensity (Figs. 1, 2), permitting bleomycin cleavage to be easily observed. The exact location of bleomycin damage in the DNA sequence was determined by direct comparison with dideoxy sequencing reactions on the same human genomic DNA sequence (Fig. 1). The DNA sequencing with dideoxyadenosine and dideoxycytidine indicate the

position of T and G, respectively, on the analysed template strand where bleomycin cleavage was observed.

This FAM-T21 oligonucleotide will bind at multiple places on the telomeric repeat. However, the extension reactions will overlap since it is being amplified on a repeat DNA sequence. Hence, the DNA sequence specificity results should be seen as a repeating six-nucleotide window on the interaction of bleomycin with telomeric DNA sequences.

Using the linear amplification technique, we detected bleomycin telomeric DNA damage in human HeLa cells. The position of bleomycin telomeric DNA damage is shown in Fig. 1. The sequence 3'-GpGpGpApTpTpGpGpGpApTpTp-5' is cleaved by bleomycin to give 3'-GpGpGpApTp-5' and 3'-pgGpGpGpApTpTp-5' (where pg is 3'-phosphoglycolate) since the T nucleotide is destroyed during bleomycin cleavage at the 3'-TpG-5' dinucleotide (underlined). Hence, the linear amplification extension will terminate opposite the T nucleotide in the bleomycin cleavage product 3'-GpGpGpApTp-5'. This is observed as the main bleomycin DNA damage site (Fig. 1). The second most intense peak is at a 3'-GG-5' dinucleotide. It is known that the linear amplification procedure with *Taq* DNA polymerase can add an extra A nucleotide to the 3' end of amplified DNA sequences [42]. This can be observed as minor peaks one nucleotide longer than the 5'-GT and 5'-GG dinucleotide bleomycin damage sites.

Hence, the results indicated that bleomycin mainly cleaves at 5'-GT dinucleotides, with lesser amounts at a 5'-GG dinucleotide (Fig. 1). The most intense site of bleomycin DNA damage in the hexamer telomeric repeat sequence is at 5'-ttaggGTta-3' (with the capital letters indicating the dinucleotide cleavage site), followed by 5'-ttaGGgta-3'.

In a comparison with human HeLa cells, purified human DNA was treated with a range of bleomycin concentrations. The results for bleomycin damage in purified DNA after linear amplification with the FAM-T21 primer are shown in Fig. 2. From 0.03 to 0.2 mM, increasing the concentration of bleomycin increased the intensity of the damage peaks. The pattern of DNA damage in telomeric sequences of purified HeLa DNA treated with 0.2 mM bleomycin and that for HeLa cells treated with 1 mM bleomycin are illustrated in Fig. 2. It can be seen that the pattern of bleomycin cleavage in purified human DNA was very similar to that found for human cells, since the major peaks occur at similar positions and the peak heights are approximately comparable (Fig. 2).

Discussion

Bleomycin is an antibiotic that is clinically used as a cancer chemotherapeutic agent [3–6]. In this work, the DNA

sequence specificity of bleomycin DNA damage in telomeric DNA sequences of human cells was established. To our knowledge, this has not been reported previously. Bleomycin preferentially cleaves DNA at sites adjacent to G nucleotides, such as GT, GA, GC and GG [35–37]. The human telomeric sequence $(GGGTTA)_n$ contains the dinucleotides 5'-GT and 5'-GG, and thus it is expected to be a strong target for the drug.

To investigate the sequence specificity of telomeric DNA damage in human HeLa cells, a linear amplification technique was used with a fluorescently labelled telomere-specific primer oligonucleotide and analysis was with CGE–LIF detection and GeneMapper. The position of the

bleomycin damage sites was determined with reference to size markers generated by dideoxy sequencing reactions on human genomic DNA. The results indicated that bleomycin preferentially cleaved at 5'-GT dinucleotides, with lesser amounts at 5'-GG dinucleotides. The 5'-ttagGTta-3' site (with the capital letters indicating the dinucleotide cleavage site) was the most intense site of bleomycin DNA damage in the telomeric sequence, followed by 5'-ttaGGgta-3'.

The DNA sequence specificity of bleomycin was similar to that reported for non-telomeric DNA sequences for purified DNA, with damage sites occurring primarily at 5'-GT dinucleotides [33, 37, 38, 43, 44]. The bleomycin telomeric DNA sequence specificity has also been determined

Fig. 1 Electropherograms of bleomycin DNA damage in telomeric repeats of HeLa cellular DNA and dideoxy DNA sequencing reactions. Electropherogram A shows the no-drug blank control. Electropherogram B depicts the telomeric damage sites for HeLa cells treated with 1 mM bleomycin. Electropherogram C depicts a dideoxyadenosine (ddA) reaction that reveals T nucleotides on the template strand, and electropherogram D depicts dideoxycytidine (ddC) and reveals G nucleotides on the template strand. The green dotted lines show the mobilities of the 5'-GT (left) and 5'-GG (right) bleomycin damage bands in comparison with the dideoxy sequencing reactions. The relative fluorescent intensity is along the y-axis and the DNA fragment size (base pairs, bp) is along the x-axis

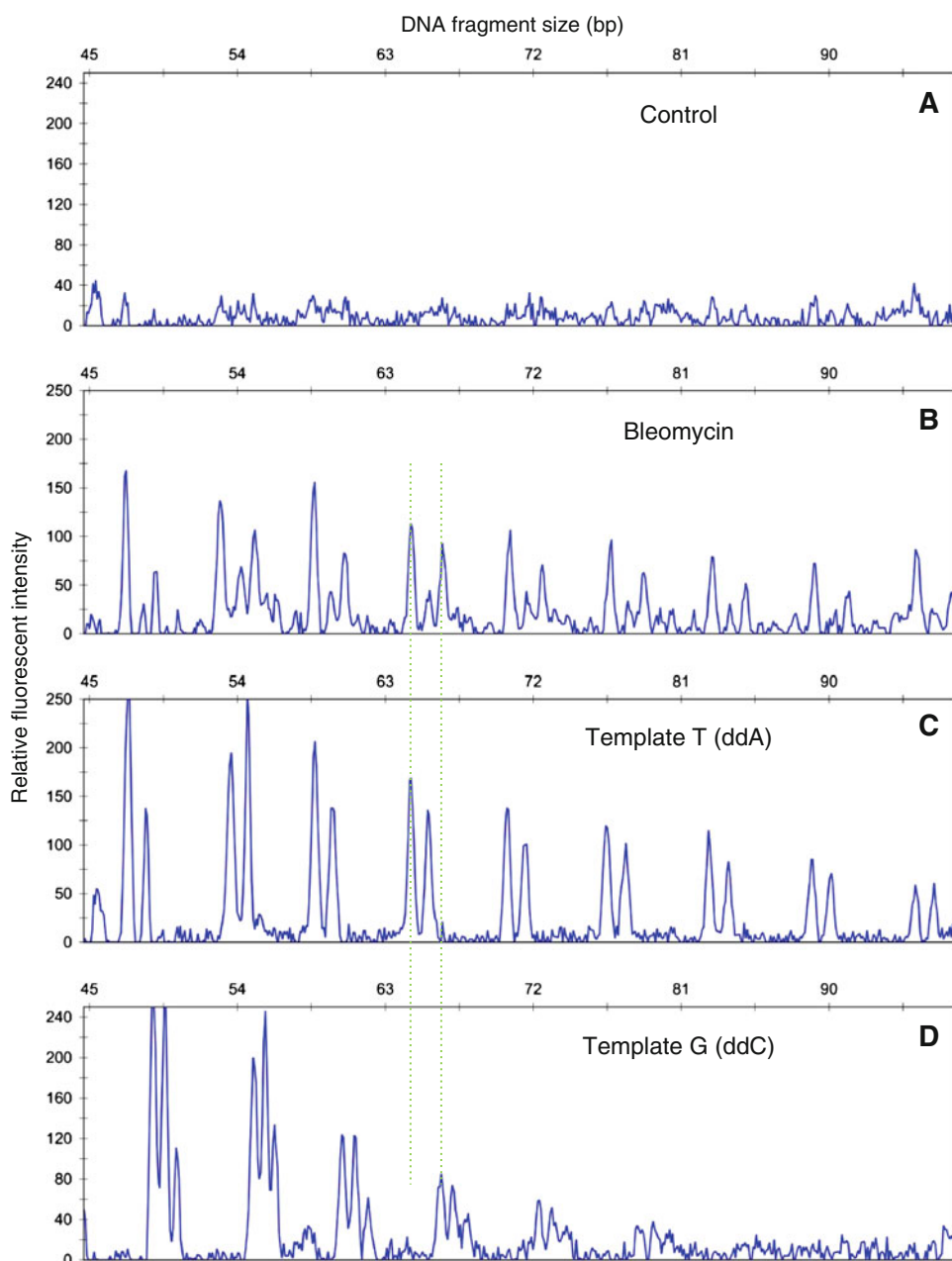
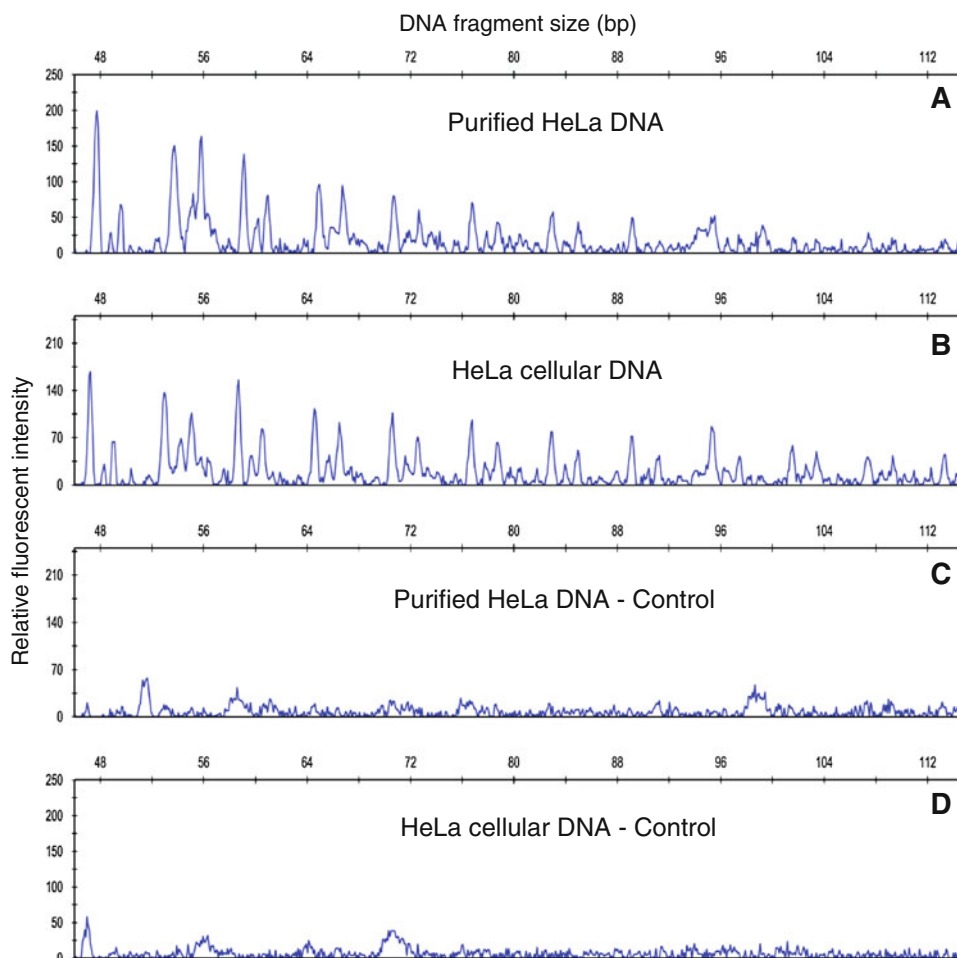


Fig. 2 Electropherograms of bleomycin cleavage in telomeric sequences of purified HeLa DNA and HeLa cellular DNA. Electropherogram *A* depicts the telomeric damage sites with 0.2 mM bleomycin in purified HeLa DNA. Electropherogram *B* depicts the telomeric damage sites with 1 mM bleomycin in HeLa cellular DNA. Electropherogram *C* is for the no-drug control (blank) for purified HeLa DNA and electropherogram *D* is for the no-drug control (blank) for HeLa cellular DNA. The relative fluorescent intensity is along the y-axis and the DNA fragment size is along the x-axis



in a purified DNA sequence containing 17 tandem telomeric repeats [33] and it was found that bleomycin preferentially targeted 5'-GT dinucleotide sequences, and to a lesser extent 5'-ttaGGgta-3' sequences, as found in this study. Previously, the bleomycin DNA sequence specificity was determined in human cells for several non-telomeric DNA sequences, including alphoid DNA [38], globin [39–41, 45] and p53 [46] but not previously for telomeric DNA sequences.

In this study, bleomycin DNA damage in HeLa cells was compared with that in purified HeLa DNA, and it was found that the prominent peaks had a similar position and intensity in both environments. This implies that the pattern of bleomycin damage in telomeric DNA sequences in intact human cells was the same as in purified DNA. This result was compatible with previous studies which have shown that there is no significant difference in the sequence specificity of DNA damage between in situ and in vitro exposure to bleomycin for alphoid DNA [38] and in the β -globin gene cluster [39].

It has been proposed that telomeres are an important target for a number of antitumour DNA-damaging agents [47–49]; however, reports are conflicting on the interaction

of bleomycin with telomeres. In experiments with purified DNA, it was found that telomeric sequences constituted most of the most intense bleomycin damage sites [33] and this indicated that telomeric sequences are major sites of bleomycin damage.

There are a number of studies with mammalian cells that support the idea of preferential bleomycin damage of telomeres compared with non-telomeric DNA sequences [50–55]. Bleomycin-induced chromosomal aberrations in human lymphocytes and Chinese hamster ovary and Chinese hamster embryo cell lines were reported to be preferentially located at telomeres [50, 51, 53–55]. Bleomycin damage of cellular telomeric sequences of Chinese hamster chromosomes was investigated by fluorescence in situ hybridisation [53]. It was demonstrated that bleomycin was able to induce telomeric damage that subsequently gave rise to chromosome breakage and recombination. It was found that telomeres were crucial for an increased level of sensitivity during bleomycin treatment in Chinese hamster embryo cells [52].

Conversely, in human lymphocytes, it was shown that the length of telomeres was not significantly different for bleomycin-treated and non-treated cells, and hence it was

concluded that bleomycin did not preferentially target telomeres [56, 57]. Arutyunyan et al. [58] used the comet assay–fluorescence in situ hybridisation technique to examine DNA fragmentation caused by bleomycin with a telomere-specific labelled peptide nucleic acid hybridisation probe. They found that the breakage frequency in telomeric DNA sequences was proportional to that of total DNA, and it was concluded that bleomycin produced DNA damage in a random manner and was not specifically targeted to telomeres.

Conclusions

The DNA sequence specificity of bleomycin damage in telomeric DNA sequences was determined in intact human cells, and this has not been reported previously. Bleomycin was found to cleave preferentially at 5'-GT dinucleotide sequences in the human telomere DNA sequence (GGGTTA)_n. There were no major differences between the sequence specificity of bleomycin damage in telomeric sequences in cellular DNA compared with purified DNA. Since telomeres have been shown to be an important site of bleomycin-induced DNA damage, this study has increased our understanding of the mechanism of action of bleomycin by determining its DNA sequence specificity in human cells. Telomeric bleomycin cleavage could be a crucial component in the activity of bleomycin as an anticancer drug, since significant damage of telomeres by bleomycin would lead to cell death.

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