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# Bleomycin analogues preferentially cleave at the transcription start sites of actively transcribed genes in human cells

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ABSTRACT

Bleomycin (BLM) is a cancer chemotherapeutic agent that is used in the treatment of several types of tumours. The cytotoxicity of three BLM analogues, BLM Z, 6′-deoxy-BLM Z and zorbamycin (ZBM), was determined in human HeLa cells in comparison with BLM. It was found that the $IC_{50}$ values were 2.9 μM for 6′-deoxy-BLM Z, 3.2 μM for BLM Z, 4.4 μM for BLM and 7.9 μM for ZBM in HeLa cells. Using next-generation Illumina DNA sequencing techniques, the genome-wide cleavage of DNA by the BLM analogues was determined in human HeLa cells and compared with BLM. It was ascertained that BLM, 6′-deoxy-BLM Z and ZBM preferentially cleaved at the transcription start sites of actively transcribed genes in human cells. The degree of preferential cleavage at the transcription start sites was quantified and an inverse correlation with the $IC_{50}$ values was observed. This indicated that the degree of preferential cleavage at transcription start sites is an important component in determining the cytotoxicity of BLM analogues.


## 1. Introduction

Bleomycin (BLM) is an anti-tumour agent that is used in the treatment of skin, head and neck carcinomas, testicular tumours and Hodgkin's lymphoma (Einhorn, 2002; Froudarakis et al., 2013; Neese et al., 2000; Stoter et al., 1994; Umezawa et al., 1966; Williams et al., 1987). The clinically-utilised BLM consists of BLM A2 (70%) and BLM B2 (30%) (Fig. 1) that are glycopeptide antibiotics and cleave DNA to produce single- and double-strand breaks as well as causing DNA base damage (Chen and Stubbe, 2005; Leitheiser et al., 2003; Rabow et al., 1990). The double-strand breaks caused by BLM are thought to be important for its anti-tumour activity (Mirabelli et al., 1985; Sikic, 1986). Its reaction mechanism involves the divalent cation $Fe^{2+}$ and generates a free radical at the 4′-position in the deoxyribose sugar of DNA that in the presence of oxygen, leads to phosphodiester bond cleavage and production of 5′-phosphate and 3′-phosphoglycolate ends at the cleavage site (Burger, 1998; Chen and Stubbe, 2005).

BLM cleaves DNA with a sequence specificity that has been defined in purified DNA sequences as being 5′-GT and 5′-GC dinucleotides (D'Andrea and Haseltine, 1978; Kross et al., 1982; Mirabelli et al., 1982; Murray, 2000; Murray and Martin, 1985a; Murray et al., 1988; Nightingale and Fox, 1993; Roy et al., 2014; Segerman et al., 2013; Takeshita et al., 1978, 1981; Tang et al., 2014). A similar DNA sequence specificity was also found in human cells (Cairns and Murray, 1996; Kim and Murray, 2000, 2001; Murray and Martin, 1985b; Nguyen and Murray, 2012; Temple et al., 2004; Temple and Murray, 2005). We recently utilised high-throughput Illumina sequencing to determine the genome-wide DNA sequence specificity of BLM in human cells and established a longer sequence preference of RTGT*ATY where T* is the cleavage site, R is a purine and Y is a pyrimidine (Murray et al., 2016).

In a previous genome-wide paper, we determined that BLM preferentially cleaves at the transcription start sites (TSSs) of actively transcribed genes (Murray et al., 2014a). In a comparison of genes that were expressed at different levels, we determined that there was a relationship between the level of gene transcription and the enhancement of BLM cleavage at the TSS. BLM preferentially cleaves in the linker region of nucleosomes (Galea and Murray, 2010; Kuo and Hsu, 1978; Murray et al., 2014a,b; Murray and Martin, 1985b). We also demonstrated that BLM could detect positioned nucleosomes at the TSS (Murray et al., 2014a).

BLM A2 and BLM B2 are produced in *S. verticillus* by a hybrid non-ribosomal peptide synthetase and polyketide synthetase biosynthetic machinery that is encoded by a large 120-kb gene cluster (Du et al., 2000; Galm et al., 2008, 2011; Shen et al.,

*Abbreviations:* BLM, bleomycin; $IC_{50}$, half maximal inhibitory concentration; MNase, micrococcal nuclease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TSS, transcription start site; ZBM, zorbamycin.

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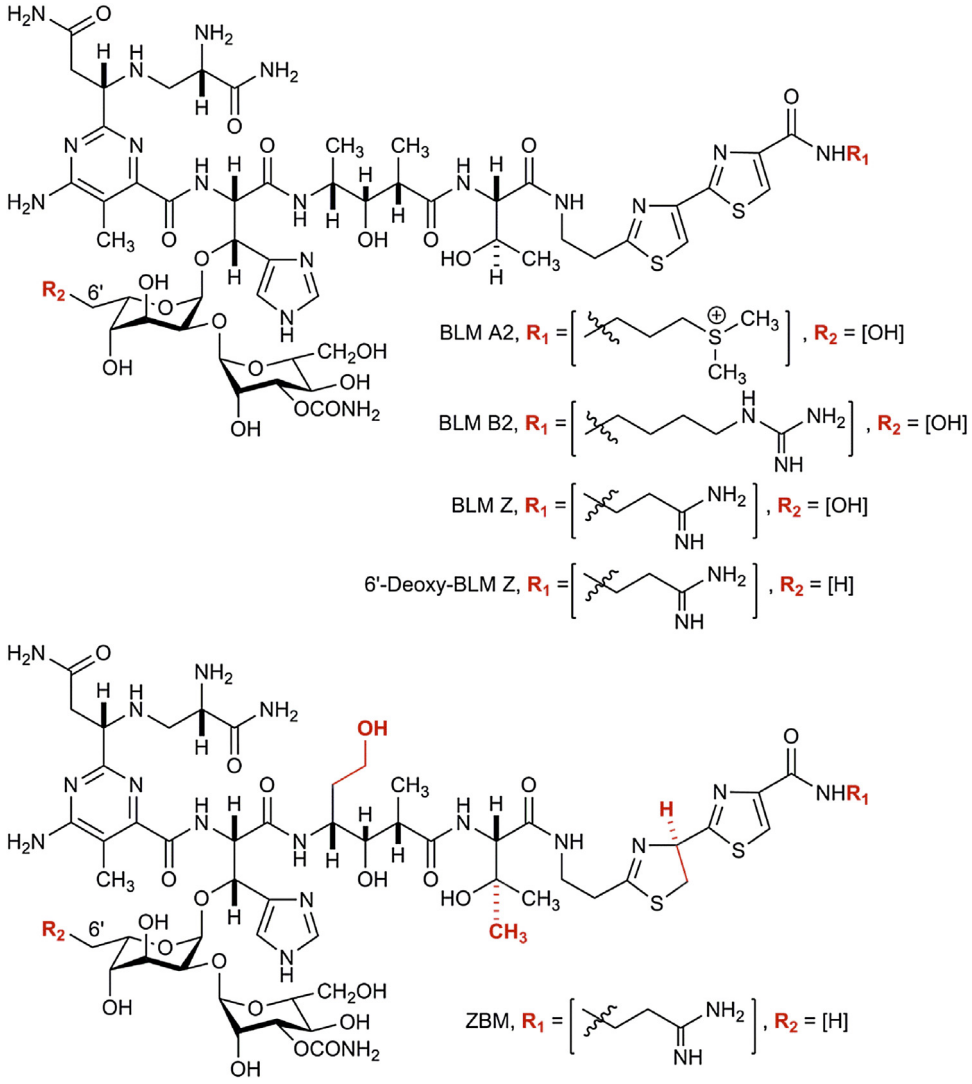


**Fig. 1.** The structures of BLM A2, BLM B2, BLM Z, 6′-deoxy-BLM Z and ZBM. Differences in chemical structure are shown in red.

2001, 2002). Although combinatorial biosynthesis has recently emerged as a promising alternative to produce structural analogues of complex natural products, engineered production of BLM analogues by manipulating BLM biosynthesis has been extremely difficult due to the genetic unamenability of the native BLM-producing strain of *S. verticillus* (Galm et al., 2008). To circumvent this problem, we have previously discovered zorbamycin (ZBM), a BLM analogue, from *Streptomyces flavoviridis* and developed *S. flavoviridis* as a heterologous host for engineered biosynthesis of novel BLM analogues, resulting in the production of two additional BLM analogues, BLM Z and 6′-deoxy-BLM Z (Huang et al., 2012). The structures of BLM, ZBM, the two engineered BLM analogues, in comparison with BLM A2 and BLM B2, are shown in Fig. 1, and they differ at a small number of positions as shown in red. Thus, while BLM Z and 6′-deoxy-BLM Z share the same C-terminal tail as ZBM, differing from BLM A2 and B2, 6′-deoxy-BLM Z shares the same disaccharide moiety as ZBM, differing from all other known BLMs, as exemplified by BLM A2, BLM B2 and BLM Z (Fig. 1) (Huang et al., 2012).

The DNA cleavage efficiency of the these BLM analogues, in comparison with BLM, has been determined, and 6′-deoxy-BLM Z was found to the most potent, followed by ZBM, BLM Z and BLM (Huang et al., 2012) (Table 1). The DNA sequence specificity of BLM Z, 6′-deoxy-BLM Z and ZBM has been elucidated in a purified plasmid sequence and compared with BLM (Chen et al., 2016). The sequence specificity of BLM, BLM Z and 6′-deoxy-BLM Z were similar; however, the sequence selectivity of ZBM was different from BLM and the other two analogues (Chen et al., 2016).

In this paper, using next-generation Illumina DNA sequencing techniques, the genome-wide cleavage of DNA by 6′-deoxy-BLM Z and ZBM was determined in human HeLa cells and compared with our previous BLM data. The technique detects double-strand breaks and these DNA cleavage sites were mapped onto HeLa cell TSSs. The degree of cleavage at the TSSs was then related to the cellular level of transcription for the two analogues and compared with BLM. This data shows the importance of genome-wide DNA sequencing where important information can be obtained concerning the impact of the analogues on the human cells. In this paper we also report the cytotoxicity of these BLM analogues and compared their properties with BLM.

## 2. Materials and methods

### 2.1. BLM and analogues

BLM was purchased from Bristol Laboratories under the trade name Blenoxane® and consisted of BLM A2 (70%) and B2 (30%). The three BLM analogues used in this study, 6′-deoxy-BLM Z, BLM Z and