ACTION OF BLEOMYCIN ON PROGRAMMED SYNTHESIS. INFLUENCE ON DNA AND RNA NUCLEASES*

Werner E.G. MÜLLER, Zen-Ichi YAMAZAKI, Jürgen E. ZÖLLNER and Rudolf K. ZAHN

Physiologisch Chemisches Institut der Universität, 65 Mainz, Saarstrasse 21, Germany

Received 8 January 1973
Revised version received 22 February 1973

1. Introduction

BLM** an antibiotic with antineoplastic activity [1] is a glycopeptide [2] produced by *Streptomyces verticillus*. It has been first isolated by Umezawa [3]. Efforts to elucidate the exact mechanism of BLM action on programmed synthesis dealt with DNA and RNA (survey: [4, 5]), with DNA and RNA polymerases [6–8] and finally with cell culture models [9, 10]. One of the main targets of BLM is the splitting off of thymine bases from natural and synthetic double stranded nucleic acids by a hydrolytic reaction. The BLM activity is enhanced by adding reducing as well as oxidizing agents. Compounds not containing thymine are not affected by BLM [5]. Ohashi [11] investigated whether the inhibition of cellular DNA synthesis by BLM may be due to an increase in DNAase activity. Umezawa and coworkers [12] in their study with *E. coli* and with HeLa cells found DNA strand scissions when BLM had been added.

On the basis of these findings it may be useful to study the influence of BLM on nuclease activity. BLM turned out to be a strong inhibitor of DNAase I. The inhibition is of the competitive type. The strength of the inhibition due to BLM is dependent from the kind of DNA used as substrate in the assay. The DNAase II is less affected by BLM modified DNA. The enzymatic activities of RNAase A, RNAase B, RNAase T_1, venom phosphodiesterase I and spleen phosphodiesterase II are not influenced by this antibiotic.

* This is part III of this series.

** *Abbreviations*:
BLM : bleomycin.
DNAase: deoxyribonuclease.
RNAase: ribonuclease.
DTT : dithiothreitol.

2. Materials and methods

Materials were obtained as follows: DNAase I (beef pancreas) 2500 units/mg, DNAase II (pig spleen) 12,000 units/mg, RNAase A (beef pancreas) 3000 units/mg, RNAase B (beef pancreas) 1000 units/mg, RNAase T_1 (*Aspergillus oryzae*) 30 000 units/mg, phosphodiesterase I (*Crotalus adamanteus*), phosphodiesterase II (beef spleen), RNA (yeast) from Worthington, Biochemical Corp., Freehold (USA); *p*-nitrophenylthymidine-5′ phosphate and Cleland's reagent (dithiothreitol) from Calbiochem, Los Angeles (USA); the different synthetic polymers from General Biochemicals, Chagrin Falls (USA); Bleomycin (lot pH 1296/G), native herring DNA, isolated according to Zahn et al. [13] from H. Mack, Illertissen (Germany).

DNAase I and DNAase II activity was determined by the spectrophotometric Kunitz method [14] using the assay conditions described by Zahn et al. [15] resp. Bernardi et al. [16]. The DNAase I reaction was initiated by the addition of 25 units of enzyme at 25°; the one of DNAase II by 20 units of enzyme. The determination of RNAase activity based upon the release of soluble oligonucleotides from RNA as described by Kalnitsky et al. [17] and Egami et al. [18]. Measurements of the activity of phosphodiesterase I were performed by the method described by

Razzel et al. [19]. The assay for determination of spleen phosphodiesterase II based on the method described by Hillmoe [20]. To the nuclease assays described above, 10 mM DTT were added.

3. Results and discussion

The enzyme reactions are performed with DTT, an agent which enhances the BLM action optimal [5]. The influence of BLM on enzyme reactions has been determined under substrate limiting conditions. This allows to detect interactions between BLM and the different substrates.

In contrast to the results of Yamaki [21], the results presented in this paper show DNAase I activity to be strongly inhibited by BLM. The inhibition has been shown to be of the competitive type (figs. 1 and 2). In the experiments summarized in fig. 1 BLM has been added to the assays at least 2 min before the beginning of the enzymatic reactions. In preceding tests it has

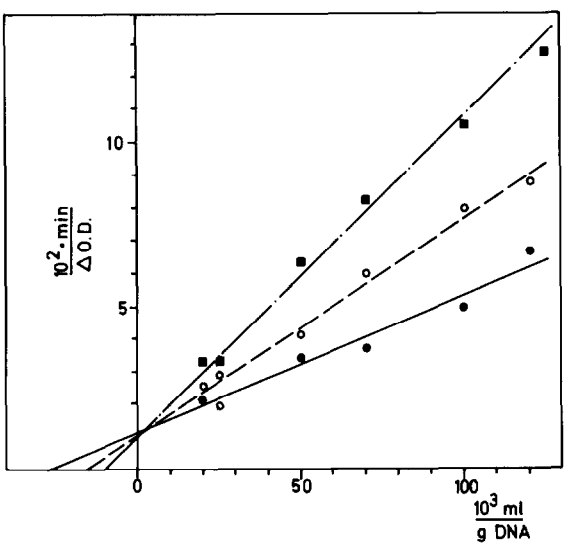

Fig. 1. Inhibition of DNAase I by BLM. Plot according to Lineweaver et al. [26]. The reaction was stopped after 4 min during the linear part of the enzyme reaction. Abscissa: reciprocal values of the substrate concentration; ordinate: Reciprocal values of the initial reaction velocity expressed as minutes per change in O.D. Linear regressions: (●—●—●) control, (○ - - ○ - - ○) 20 μg BLM/ml and (■ - - - ■) μg BLM/ml. K_i = 31.8 ±9.2 μg BLM/ml, K_m = 38.5 ± 10.5 μg DNA/ml.

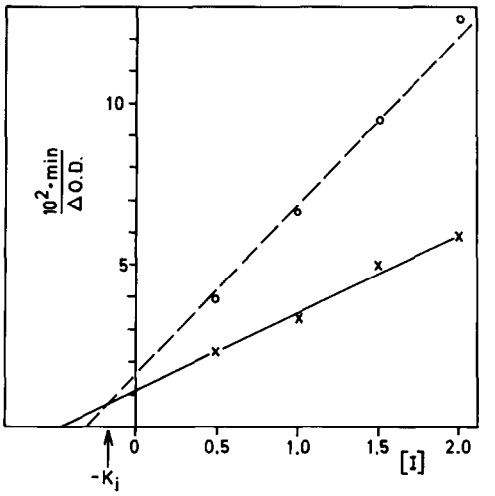

Fig. 2. Influence of poly (dA–dT), modified by BLM and DTT on DNAase I reaction. 1 mg poly (dA–dT) was modified in a 1.0 ml assay during a reaction (15 min, 37°) with 25 μg BLM/ml and 5 mM DTT, in a 40 mM Tris buffer (pH 7.8). After the incubation, the mixture was dialyzed for 16 hr against 2 × 500 ml Tris buffer mentioned. Subsequently the modified poly (dA–dT) was added at different concentrations (as indicated on the abscissa in μg/ml) to the enzyme mixture. The reaction was stopped after 4 min. X—X—X 90 μg DNA/ml; ○—○—○ 35 μg DNA/ml. Plot according to Dixon [24]. K_m = 47.1 ± 8.6 μg DNA/ml; K_i = 0.17 ± 0.03 μg BLM-modified poly (dA–dT)/ml. Ordinate: reciprocal values of the initial reaction velocity expressed as minutes per change in O.D.

been found that the inhibitory influence exerted by BLM on the enzymatic reaction occurs so fast that it can not be detected. The inhibitor constant using native DNA as substrate is determined to be 31.8 μg BLM/ml (fig. 1). Basing on a comparison of BLM influence to the hydrolytic activity of DNAase I using different DNA substrates it is evident that the structure of the DNA is crucial for the activity of the compound. With application of single stranded homopolymers (poly (dA) and poly (dT)) no inhibitory influence can be observed. Regarding the double stranded polymers tested, native herring DNA, poly (dA·dT), poly (dA–dT) and poly (dG·dC), only thymine containing DNA's produce with BLM a decrease of the enzyme activity. Remarkable is the finding that with the copolymer poly (dA–dT) the BLM inhibitory influence on DNAase I is 3.4 times higher than with the homopolymer poly (dA·dT). This finding coincides with the

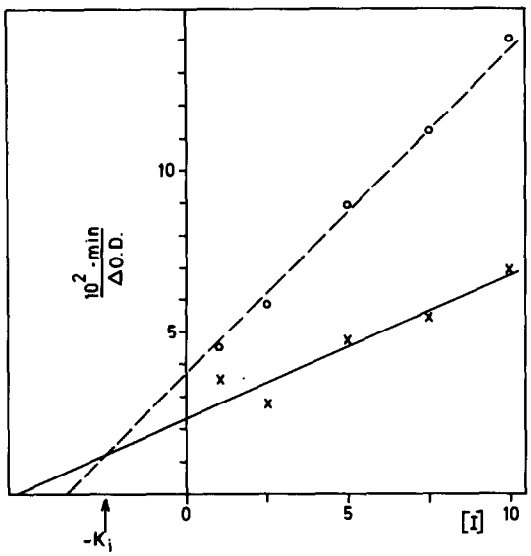

Fig. 3. Influence of poly (dA–dT), modified by BLM and DTT on DNAase II reaction. Plot according to Dixon [24]. Abscissa: concentration (in μg/ml) of modified poly (dA–dT) in the standard enzyme mixture. Ordinate: reciprocal values of the initial reaction velocity expressed as minutes per change in O.D. The reaction was terminated after 10 min during the linear part of the enzyme reaction. ×—×—× 130 μg DNA/ml; ○—○—○ 65 μg DNA/ml; K_m = 128.7 ± 29.0 μg DNA/ml; K_i = 2.5 ± 0.8 μg BLM-modified poly (dA–dT)/ml. For further details see legend fig. 2.

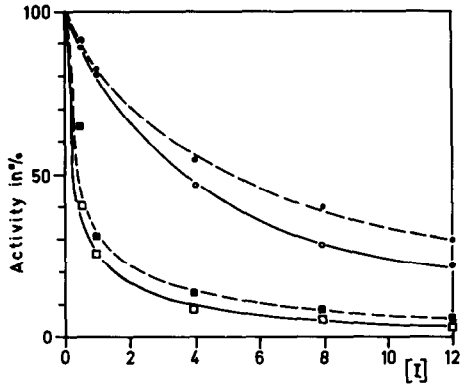

Fig. 4. Influence of poly (dA–dT), modified by BLM and DTT on DNAase I and DNAase II reaction. Abscissa: concentration (in μg/ml) of modified poly (dA–dT) in the enzyme mixtures. Ordinate: activity of the enzyme in percent. Substrate concentration in DNAase I experiments were 47 μg DNA/ml (□—□—□) and 129 μg DNA/ml (■—■—■) resp. for DNAase II 47 μg DNA/ml (○—○—○) and 129 μg DNA/ml (●—●—●). For further details see legend to fig. 2.

experiments measuring the thymine liberation activity of BLM [5]: under identical conditions 3.2 times more thymine are liberated using poly (dA–dT) than with poly (dA·dT). In other experiments (fig. 2) it is demonstrated that the inhibitory influence of BLM on DNAase I is due to a BLM caused modification of the substrate. For this set of experiments poly (dA–dT) was preincubated with BLM and DTT. In a subsequent procedure BLM and DTT were removed

Table 1
Quantitative comparison of BLM action on different DNA and RNA nucleases.

Enzymes	Source of the enzyme	Substrate concentration (per ml)	BLM concentration (± S.D.) causing 50% inhibition (μg/ml)
Ribonuclease A	Beef pancreas	100 μg RNA	No inhibition
Ribonuclease B	Beef pancreas	100 μg RNA	No inhibition
Ribonuclease T_1	*Aspergillus oryzae*	100 μg RNA	No inhibition
Phosphodiesterase I	*Crotalus adamanteus*	200 μg nitrophenyl-thymidine-phosphate	No inhibition
Phosphodiesterase II	Beef spleen	100 μg RNA	No inhibition

The BLM concentration that causes a 50% reduction of enzyme activity was been determined under substrate conditions indicated in table. The results of eight independent experimental series are given with their standard deviations. The enzyme concentrations were as follows (μg resp. units per ml): RNAase A: 2.5 μg; RNAase B: 2.5 μg; RNAase T_1: 0.025 μg; phosphodiesterase I: 100 μg and phosphodiesterase II: 0.25 units.

from poly (dA–dT) by dialysis; this separation can be followed spectrophotometrically. The modified poly (dA–dT) preparation was added at different concentrations to the enzyme assay containing two different substrate (DNA) concentrations. The results are plotted according to Dixon [22]. Applying this plot, a competitive type of inhibition could also be determined, a fact which indicates that the decrease of enzymatic depolymerization in the assays with BLM as well as with BLM modified poly (dA–dT) is due to a decrease of the affinity of the enzyme to the modified substrate.

Thus it can be ruled out that an interaction takes place between the inhibitor and the enzyme. In this view, the mechanism of inhibition of DNAase I reaction by BLM-modified DNA seems to have similar properties as the enzyme reaction using apurinic acid substrate [23].

DNAase II is inhibited by BLM modified poly (dA–dT) also in a competitive way. However, the DNAase II activity is much less affected by BLM than the DNAase I reaction. This result is not caused by a possible pH dependent BLM action as shown by us [5]. The reason may be seen in a 15-fold higher inhibitor constant for BLM modified DNA in the case of DNAase II (fig. 3) compared to the reaction with DNAase I. This conclusion is supported by a comparison of the inhibitory action of BLM modified poly (dA–dT) on DNAase I and DNAase II (fig. 4) using DNA substrate concentrations of 47 μg/ml, resp. 129 μg/ml which represent the Michaelis constants of the two enzymes for DNA (figs. 2 and 3). On an average (fig. 4) DNAase I reactions are inhibited by BLM modified poly (dA–dT) 30-fold stronger than that of DNAase II. This finding seems to be in agreement with the DNAase II characteristic [24, 25] to favour the cleavage of d-Gp–Cp linkages in DNA.

The reactions of the *RNAases (A, B and T_1) as well* as of two *phosphodiesterases (venom and spleen)* by using RNA and *p*-nitrophenylthymidine-5′ phosphate as substrates are not inhibited by BLM up to a concentration of 200 μg/ml (table 1). The results [5] showing no splitting activity of BLM towards RNA exclude the possibility to influence the enzymatic hydrolysis by an attack on the substrate. It is also unlikely that *p*-nitrophenylthymidine-5′ phosphate is modified by BLM because only double stranded natural or synthetic DNA's are affected by this antibiotic. Thus the results presented in this report demonstrate clearly BLM to be not an enzyme poison for nucleases.

Acknowledgements

We thank H. Mack, Illertissen (Germany), for gifts of bleomycin and herring DNA. The authors express their gratitude to Mr. R. Beyer and D. Sabol.

References

[1] H. Satoh and H. Ichimura, Igaku no Ayumi (Progress in Medicine) 69 (1969) 669.
[2] T. Takita, K. Macda, H. Umezawa, S. Omoto and S. Umezawa, J. Antibiotics 22 (1969) 237.
[3] H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, J. Antibiotics 19 (1966) 200.
[4] I. Shirakawa, M. Azegami, S. Ishii and H. Umezawa, J. Antibiotics 24 (1971) 761.
[5] W.E.G. Müller, Z. Yamazaki, H.J. Breter and R.K. Zahn, European J. Biochem. 31 (1972) 518.
[6] Z. Yamazaki, W.E.G. Müller and R.K. Zahn, Biochim. Biophys. Acta, in press.
[7] W.E.G. Müller, Z. Yamazaki and R.K. Zahn, Biochem. Biophys. Res. Commun. 46 (1972) 1667.
[8] N. Tanaka, J. Antibiotics 23 (1970) 523.
[9] H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka and H. Umezawa, J. Antibiotucs 21 (1968) 379.
[10] S.C. Barranco and R.M. Humphrey, Cancer Res. 31 (1971) 1218.
[11] S. Ohashi, Symposium of a new anticancer drug bleomycin. From pathological viewpoint. The 6th Congress of Japan Society for Cancer Therapy, Oct. 17, 1968.
[12] H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka and H. Umezawa, J. Antibiotics 22 (1969) 446.
[13] R.K. Zahn, E. Tiesler, A.K. Kleinschmidt and D. Lang. Biochem. Z. 336 (1962) 281.
[14] M. Kunitz, J. Gen. Physiol. 33 (1950) 349.
[15] R.K. Zahn, E. Tiesler, H.G. Ochs, B. Heicke, W. Forster, W. Hanske and D. Lang, Biochem. Z. 341 (1965) 502.
[16] G. Bernardi and M. Griffe, Biochemistry 3 (1964) 1419.
[17] G. Kalnitsky, J.P. Hummel and C. Dierkes, J. Biol. Chem. 234 (1959) 1512.
[18] F. Egami, K. Takahashi and T. Uchida, in: Progress in nucleic acid research and molecular biology, Vol. 3, eds. J.N. Davidson and W.E. Cohn (Academic Press, New York, 1964) p. 59.
[19] W.E. Razzell and H.G. Khorana, J. Biol. Chem. 234 (1959) 2105.
[20] R.J. Hilmoe, Spleen Phosphodiesterase, in: Biochemical Preparations, Vol. 8, ed. A. Meister (John Wiley and Sons, New York, 1961) p. 105.
[21] H. Yamaki, H. Suzuki, K. Nagai, N. Tanaka and H. Umezawa, J. Antibiotics 24 (1971) 178.

[22] M. Dixon, Biochem. J. 55 (1953) 170.
[23] C. Tamm, H.S. Shapiro and E. Chargaff, J. Biol. Chem. 199 (1952) 314.
[24] S. Vanecko and M. Laskowski, J. Biol. 236 (1961) 1135.
[25] J. Doskocil and E. Sorm, Biochim. Biophys. Acta 48 (1961) 211.
[26] H. Lineweaver and D. Burke, J. Am. Chem. Soc. 56 (1934) 408.