

DNA Breaks Induction by Mimosine

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Mouse erythroleukemic F4 N cells were treated with mimosine, etoposide, Fe(II)-EDTA, and Cu(II) in the presence of ascorbate. DNA was isolated and subjected to agarose gel electrophoresis and the size and distribution of the DNA fragments produced by the agents were compared. With increasing concentration of Cu(II) the production of DNA fragments was increased without decrease of the average length of the fragments, and their sizes were similar to those produced by etoposide as expected for cleavage of DNA at the nuclear matrix attachments sites. In contrast, mimosine and Fe(II) produced fragments of random size and with the progression of the reaction the average length of the fragments decreased. These results indicate that mimosine cuts DNA in a random fashion, regardless of its higher order chromatin organization. A conclusion is drawn that the DNA fragments obtained after mimosine treatment are a result of mimosine-assisted, Fe(II) dependent Fenton-like reactions randomly cutting chromosomal DNA.

Key words: Mimosine, DNA Breaks, Nuclear Matrix

Mimosine is an inhibitor of DNA synthesis that is widely used as an agent for synchronization of mammalian cells at the G1/S phase boundary of the cell cycle. That is why a considerable effort has been made to elucidate its mechanism of action (Gilbert *et al.*, 1995; Alpan and Pardee, 1996; Kalejta and Hamlin, 1997; Tsvetkov *et al.*, 1997; Ji *et al.*, 1997; Krude, 1999; Saintigny and Lopez 2002). It has been shown that mimosine has clastogenic effect, causes apoptosis and introduces DNA breaks independently of the metabolic activity of DNA (Jha *et al.*, 1995; Mikhailov *et al.*, 2000b; Saintigny *et al.*, 2001). Mimosine represents β -[N-(3-hydroxy-4-pyridone)]- α -amino-propionic acid and the ketohydroxy metal-chelating site on the pyridine ring indicates that a part of the complex action of mimosine may be a result of metal complexing. Cells treated with mimosine undergo changes in their chromatin organization and a hypothesis has been put forward that they may be triggered by the chelation of Cu(II) ions by mimosine at the nuclear matrix attachment sites (Vogt, 1991). Recently, we have shown that the mimosine-assisted DNA cleavage may be a result of its ability to form complexes with transition metals thus causing oxidative damage of DNA by Fenton-like reactions (Mikhailov *et al.*, 2000a). Copper ions play a crucial role in higher order

chromatin structure (Kaufmann *et al.*, 1981; Lewis and Laemmli, 1982). This is presumed to be achieved by stabilization of the association between the bases of DNA loops and topoisomerase II, a major component of the nuclear matrix (Earnshaw and Heck, 1995; Earnshaw *et al.*, 1985; Gasser *et al.*, 1986; Gasser *et al.*, 1989). Thus it could be suggested that mimosine introduces DNA breaks at the matrix-attachment sites *via* oxidative reactions upon association with copper ions. To check this possibility we compared the production of DNA fragments after *in vivo* treatment of cells with mimosine, the inhibitor of topoisomerase II etoposide, Fe(II)-EDTA and Cu(II). DNA was subjected to agarose gel electrophoresis and the size and distribution of the DNA fragments produced by the agents compared. The results do not support a preferential binding of mimosine with copper ions associated with the nuclear matrix, but rather indicate that mimosine produces random cleavage of DNA similar to that caused by Fe(II)-EDTA.

Materials and Methods

Cell treatment

Mouse erythroleukemia cells, clone F4 N (Dube *et al.*, 1975) were cultured in MEM-S medium sup-

plemented with 10% fetal calf serum. 10 mM stock solution of mimosine (β -[N-(3-hydroxy-4-pyridone)]- α -amino-propionic acid) was prepared in MEM-S medium and added to the cell cultures for 2 to 12 h in a final concentration of 400 μ M. 5 mM stock solution of etoposide (4'-desmethylepipodophyllotoxin-9-[4,6-O-ethylidene]- β -D-glucopyranoside) was prepared in water and added to the cell cultures to a final concentration of 60 μ M for 1 h.

Treatment of the cells with transition metals was carried out after immobilization of the cells in low-melting agarose plugs. Fe(II)-EDTA was freshly prepared by mixing equal volumes of 10 mM Fe(NH₄)₂(SO₄)₂ and 16 mM EDTA. The final concentrations of Fe(II) used were 0.5 to 5 mM and treatment was carried out for 1 h on ice. Treatment with Cu(II) was carried out with 35 to 350 μ M CuSO₄ in the presence of 0.2 mM ascorbate for 2 h on ice.

Isolation of DNA and electrophoresis

About 10⁷ exponentially growing F₄N cells were collected by low speed centrifugation, washed in phosphate buffered saline and suspended in 0.25 ml 50 mM Tris (Tris[hydroxymethyl]amino-methane), 50 mM EDTA pH 7.8 and gently mixed with equal volume of 2% low-melting agarose prepared in the above buffer. The agarose plugs were treated with lysis buffer containing 1% SDS, 1 M NaCl 50 mM Tris, 50 mM EDTA, pH 7.8, and 0.1 mg/ml Proteinase K for 24 h at 37 °C. The plugs were washed 3 times for 1 hour with 50 mM Tris, 50 mM EDTA, pH 7.8 at 4 °C, after which treated with 0.1 mg/ml RNase A for 2 h at 37 °C. Gel electrophoresis was performed in 0.5% agarose gel in 2 mM EDTA, 40 mM Tris acetate, pH 8.0 for 18 h at 1 V/cm and stained with ethidium bromide. As a molecular weight marker, λ DNA with molecular weight 48.5 kb was used. The plugs containing etoposide treated cells immediately before electrophoresis were soaked for 1 hour in 30 mM NaOH, 10 mM EDTA.

Results and Discussion

Recently we have performed *in vitro* experiments showing that mimosine (β -[N-(3-hydroxy-4-pyridone)]- α -amino-propionic acid) can enhance the cleavage reaction of DNA by transition metal ions and oxidative species, suggesting that this

could be the mechanism of mimosine-assisted introduction of DNA breaks *in vivo* (Mikhailov *et al.*, 2000a). It has been shown that treatment of nuclei with iron ions induced random double-strand DNA breaks as judged by the size distribution of DNA fragments in pulse-field gel electrophoresis. In contrast, treatment with Cu(II) produced uniform size (about 100–200 kbp) DNA fragments, independent of the yield of double-strand breaks. These observations suggested that double-strand breaks were induced by oxidative damage of DNA by Fenton-like reactions at sites of metal ion binding. These sites differed for iron and copper ions, with iron ions binding nonspecifically to exposed regions of DNA and copper ions binding to non-histone proteins at the nuclear matrix attachment sites (Chiu *et al.*, 1995). To elucidate the mechanism of mimosine induced DNA breaks formation we compared the fragment size distribution produced by mimosine treatment *in vivo* with that produced by iron and copper ions. Our previous results have shown that treatment with 400 μ M mimosine effectively inhibited DNA synthesis at the second hour of treatment, but the rate of break accumulation increased with the time of incubation (Mikhailov *et al.*, 2000b). Exponentially growing F₄N cells were treated with 400 μ M mimosine for 2, 8, and 12 h. In order to minimize the formation of DNA breaks due to shearing during the isolation procedure, isolation of DNA was performed after the cells were embedded in low melting agarose. To resolve the high

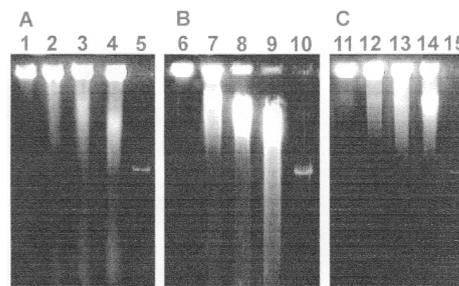


Fig. 1. Agarose gel electrophoresis of DNA isolated from F₄N cells treated with mimosine – A, Fe(II)-EDTA – B, and CuSO₄ in the presence of ascorbate – C. Lanes 2 to 4 – cells were treated with 400 μ M mimosine for 2, 8, and 12 h, respectively; lanes 7 to 9 – cells treated with 0.5, 1.5, and 5 mM Fe(II); lanes 12 to 14 – cells treated with 35, 100, and 350 μ M Cu(II); lanes 1, 6, and 11 – control untreated cells; lanes 5, 10, and 15 – λ DNA.

molecular weight DNA fragments electrophoresis was carried out in low percentage agarose and at low voltage conditions. The results presented on Fig. 1A show that the rate of break accumulation increases with the time of incubation and the molecular weight of the fragments gradually decreases. This result confirmed our previous results obtained with single cell gel electrophoresis (Mikhailov *et al.*, 2000b). The fact that with the progression of the cleavage reaction the average length of the DNA fragments decreased indicates that mimosine produces random cleavage of DNA. The treatment of the cells with metal ions was performed after permeabilization in low ionic strength buffer and inclusion in low melting agarose. In agreement with the results of Chiu *et al.* (1995) Fe(II)-EDTA produced DNA fragments with decreasing sizes (Fig. 1B), while treatment with increasing concentrations with Cu(II) in the presence of ascorbate produced uniform size DNA fragments, independent of the yield of DNA breaks (Fig. 1C).

Etoposide is an inhibitor of DNA synthesis known to block the religation step of DNA topoisomerase II and produces enzyme-linked double strand breaks. The enzyme is involved in completing DNA synthesis at the end of the replicons and is a major component of the nuclear matrix. Analysis of the long-range distribution of topoisomerase II-mediated double strand cleavages induced by etoposide have shown that the primary target of the drug *in vivo* is topoisomerase II associated with the nuclear matrix (Gromova *et al.*, 1995; Iarovaia *et al.*, 1996; Miassod *et al.*, 1997).

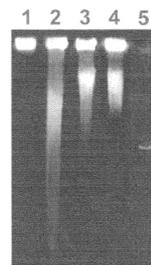


Fig. 2. Agarose gel electrophoresis of DNA isolated from F4 N cells treated with mimosine, Fe(II)-EDTA, CuSO₄ in the presence of ascorbate and etoposide. Lane 1 – control untreated cells; lane 2 – cells treated with 400 μM mimosine for 12 h; lane 3 – cells treated with 350 μM Cu(II); lane 4 – cells treated with 60 μM etoposide; lane 5 – λ DNA.

Thus it was interesting to compare the size distribution of the fragments produced by mimosine, Cu(II) and etoposide. The cells were treated *in vivo* with 60 μM etoposide, a concentration that effectively inhibits DNA synthesis after 1 hour of incubation (Kunnev *et al.*, 2000). The results are presented by Fig. 2. As it was expected the fragments produced by Cu(II) and etoposide are of similar size, indicating that the two agents are acting at the same sites of chromatin – the matrix attachment sites, while the fragments produced by mimosine were of lower molecular weight.

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- Alpan R. and Pardee A. (1996), p21^{WAF1/CIP1SD11} is elevated through a p53-independent pathway by mimosine. *Cell Growth Different.* **7**, 893–901.
- Chiu S., Xue L., Friedman L. R., and Oleinick N. L. (1995), Differential dependence on chromatin structure for copper and iron ion induction of DNA double-strand breaks. *Biochem.* **34**, 2653–2661.
- Dube S. K., Pragnell I. B., Kluge N., Gaedicke G., Stein-Heider G., and Ostertag W. (1975), Induction of endogenous and of spleen focus-forming viruses during dimethylsulfoxide-induced differentiation of mouse erythroleukemia cells transformed by spleen focus-forming virus. *Proc. Natl. Acad. Sci. USA* **72**, 1863–1867.
- Earnshaw W. C. and Heck M. M. (1985), Localization of topoisomerase II in mitotic chromosomes. *J. Cell Biol.* **100**, 1716–25.
- Earnshaw W. C., Halligan B., Cooke C. A., Heck M. M., and Liu L. F. J. (1985), Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* **100**, 1706–15.
- Gasser S. M., Laroche T., Falquet J., Boy de la Tour E., and Laemmli U. K. (1986), Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* **188**, 613–29.
- Gasser S. M., Amati B. B., Cardenas M. E., and Hofmann J. F. (1989), Studies on scaffold attachment sites and their relation to genome function. *Int. Rev. Cytol.* **119**, 57–96.
- Gilbert D., Neilson A., Miyazawa H., DePamphilis M., and W. Burhans. (1995), Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. *J. Biol. Chem.* **270**, 9597–9606.
- Gromova I. I., Thomsen B., and Razin S. V. (1995), Different topoisomerase II antitumor drugs direct similar specific long-range fragmentation of an amplified c-MYC gene locus in living cells and in high-salt-extracted nuclei. *Proc. Natl. Acad. Sci. USA* **92**, 102–106.
- Iarovaia O., Hancock R., Lagarkova M., Miassod R., and Razin S. (1996), Mapping of genomic DNA loop organization in a 500-kilobase region of the *Drosophila* X chromosome by the topoisomerase II-mediated DNA loop excision protocol. *Mol. Cell. Biol.* **16**, 302–308.
- Jha A., Hande P., Mullenders L. H. F., and Natarajan A. (1995), Mimosine is a potent clastogen in primary and transformed hamster fibroblasts but not in primary and transformed human lymphocytes. *Mutagenesis* **10**, 385–2921.
- Ji O., Marnett L. J., and Pietenpol J. A. (1997), Cell cycle re-entry following chemically induced synchronization leads to elevated p53 and p21 protein levels. *Oncogene* **15**, 2749–2753.
- Kalejta R and Hamlin J. (1997), The dual effect of mimosine on DNA replication. *Exp. Cell. Res.* **231**, 173–183.
- Kaufmann S. H., Coffey D. S., and Shaper J. H. (1981), Considerations in the isolation of rat liver nuclear matrix, nuclear envelope, and pore complex lamina. *Exp. Cell Res.* **132**, 105–23.
- Krude T. (1999), Mimosine arrests proliferating human cells before onset of DNA replication in a dose dependent manner. *Exp. Cell Res.* **247**, 148–159.
- Kunnev D., Gospodinov A., Russev G., and Anachkova B. (2000), Regulation of DNA synthesis by the higher-order chromatin structure. *DNA Cell Biol.* **19**, 283–90.
- Lewis C. D. and Laemmli U. K. (1982), Higher order metaphase chromosome structure: evidence for metalloprotein interactions. *Cell* **29**, 171–81.
- Miassod R., Razin S., and Hancock, R. (1997), Distribution of topoisomerase II-mediated cleavage sites and relation to structural and functional landmarks in 830 kb of *Drosophila* DNA. *Nucl. Acid. Res.* **25**, 2041–2046.
- Mikhailov I., Ninova P., Russev G., and Anachkova B. (2000a), Iron(II)-mimosine catalyzed cleavage of DNA. *Z Naturforsch. [C]*. **55**, 849–51.
- Mikhailov I., Russev G., and Anachkova, B. (2000b), Treatment of mammalian cells with mimosine generates DNA breaks. *Mutat. Res.* **459**, 299–306.
- Tsvetkov L., Russev, G. and Anachkova B. (1997), Effect of mimosine on DNA synthesis in mammalian cells. *Cancer Res.* **57**, 2252–2255.
- Vogt G. (1991), *In vivo* decondensation of chromatin and nuclear fibrillar component by *Leucaena leucocephala* ingredient. *Biol. Cell* **72**, 211–215.