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Effect of Mimosine on DNA Synthesis in Mammalian Cells[1]

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ABSTRACT

We have designed a general protocol to assess the rate of replicon initiation in mammalian cells in the presence of inhibitors of DNA synthesis. It is based on cross-linking DNA *in vivo* with trioxsalen, which effectively blocks the movement of the replication forks along DNA, while having little effect on initiation of replication. We applied this protocol to study the effect of the plant amino acid mimosine on the rate of replicon initiation in exponentially growing murine erythroleukemia F_4N cells. We found out that during the first 2 h after application of 25–400 μM mimosine, the initiation step was inhibited more efficiently than the overall DNA synthesis. In this respect, the effect of mimosine was similar to that of γ-ray irradiation and differed from that of hydroxyurea and aphidicolin. The results suggest that in addition to inhibiting the elongation step of DNA synthesis, mimosine inhibits the initiation of DNA replication as well.

INTRODUCTION

Chemicals that affect DNA synthesis are potentially useful for the development of anticancer drugs and are effective tools for investigation of DNA metabolism. Studies of the initiation of DNA synthesis in mammalian cells have been hampered by the lack of drugs that specifically prevent either entry into the S-phase or initiation at replication origins. That is why the interest toward the amino acid mimosine, reported to be an effective inhibitor of DNA synthesis in mammalian cells, is steadily growing. However, there are conflicting results concerning the mechanism of its action on DNA synthesis. Mimosine, β-[*N*-(3-hydroxy-4-pyridone)]-α-amino-propionic acid, is a rare nonprotein amino acid, derived from *Mimosa* and *Leucaena* plants. It was reported to be a specific inhibitor of the G_1-S phase transition of the cell cycle (1, 2). If mimosine was to arrest cells immediately before the S-phase, it would be a powerful tool for studies of the initiation of DNA synthesis, and this possibility was examined in some detail. It was shown that mimosine arrested progression through the cell cycle only after the cells entered the S-phase and DNA synthesis had begun (3). Mimosine inhibited DNA replication throughout the S-phase, and its inhibitory effect was higher when added in the first half of the S-phase than when added later in the S-phase. When added to asynchronous cell cultures, mimosine exhibited the slow-stop phenotype that characterized initiation mutants in bacteria (4, 5). Examination of the pattern of replication fork intermediates during treatment with mimosine by the method of two-dimensional gel electrophoresis showed that mimosine prevented formation of new replication forks, while allowing previously existing forks to complete replication (6). On the basis of these observations, it was suggested that mimosine might be a specific inhibitor of the initiation of DNA replication. However, other results argued that mimosine arrested DNA synthesis at the elongation step rather than at the initiation step of DNA replication. Thus, it was shown that mimosine inhibited mitochondrial, cellular, and SV40 DNA synthesis to a similar extent, despite the fundamental differences in the way replication occurred in these chromosomes (7). Mimosine was found to exhibit no effect on initiation and elongation of replication in *Xenopus* eggs or egg extracts that contain high levels of deoxyribonucleotide triphosphates and in lysates of mammalian cells supplied with exogenous deoxyribonucleotide triphosphates (7, 8). In these systems the inhibitory effect of mimosine was similar to that of hydroxyurea, which affected the levels of deoxyribonucleotide triphosphate pools in the cell. Recently, it was found that mimosine could be specifically photocross-linked to a 50-kDa polypeptide, which was 77.5% identical and 96.5% similar to mitochondrial serine hydroxymethyltransferase, and it was shown that mimosine might inhibit both the mitochondrial and cytosolic forms of the enzyme (9, 10). The latter is involved in the penultimate step of thymidylate biosynthesis in mammalian cells and therefore it could be expected that mimosine would inhibit replication by lowering the cellular deoxyribonucleotide pools.

To solve the apparent contradiction in the literature concerning the effect of mimosine on DNA synthesis, we applied the recently developed protocol for quantification of replicon initiation rates to mimosine. The protocol is based on cross-linking DNA *in vivo* with trioxsalen. The cross-links effectively block the movement of the replication fork, *i.e.*, the process of elongation, while having little effect on replicon initiation. The results show that at low doses and in the early hours after administration, mimosine inhibits initiation of DNA replication.

MATERIALS AND METHODS

Cells, Drug Treatment, and Irradiation. Friend erythroleukemia cells, clone F_4N, were cultured in MEM-S medium supplemented with 10% calf serum. The cells were treated with either (*a*) increasing concentrations of mimosine (25–400 μM) for 1 h; (*b*) 200 μM mimosine for 20, 40, 60, 90, and 120 min; (*c*) 0.6 μM aphidicolin for 20 to 120 min; (*d*) 5 mM hydroxyurea for 20 to 120 min; or (*e*) irradiated with a ^{60}Co γ source at a rate of 1 Gy/min in an ice bath to receive doses of up to 10 Gy.

Labeling of the Cells. To uniformly label DNA, prior to treatment with inhibitors, the cells were grown with 0.025 μCi of [^{14}C]thymidine for 24 h. To determine the rate of synthesis of DNA, aliquots of 2×10^6 cells for each dose of the inhibitors and time point were labeled with 1 μCi/ml of [^{3}H]thymidine for 30 min without removing the drugs from the medium. To determine the rate of initiation of DNA synthesis, aliquots of 4×10^6 cells for each dose and time point were cross-linked with trioxsalen in the presence of the drugs. Trioxsalen was dissolved in ethanol to prepare a stock solution of 0.5 mg/ml and 5 μl/ml of this stock solution were added to the cell suspension in an ice bath. After 2 min in the dark, cells were exposed to a UV lamp with two 15-W low-pressure tubes with an emission maximum at 354 nm for 2 min at 12 cm distance. The procedure was repeated twice to give 2–3 kb between the cross-links (11). After the cross-linking, 5 μCi/ml [^{3}H]thymidine (specific activity, 87.7 Ci/mmol) were added and DNA was labeled for 45 min at 37°C.

Isolation of DNA, Electrophoresis, and Counting. To isolate high molecular mass genomic DNA, cells were washed twice with 0.14 M NaCl and 0.01 M phosphate buffer (pH 7.3) and lysed in 1 M NaCl, 1% SDS, 20 mM EDTA, and 50 mM Tris-HCl (pH 7). Proteins were digested with 200 μg/ml Proteinase K overnight at 37°C and DNA was extracted with phenol-chloroform, 1:1 (once), chloroform-isoamyl alcohol, 24:1 (once), and precipitated with ethanol. DNA was dissolved in 10 mM phosphate buffer (pH 7), and its specific radioactivity was determined by reading the absorbance at 260 nm and measuring the radioactivity. DNA was denatured in glyoxal-DMSO mixture at

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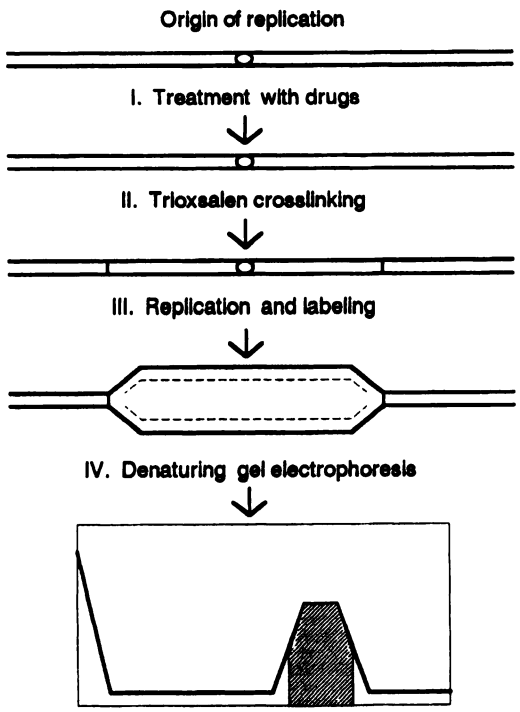

Fig. 1. Diagram of the experimental approach. *I*, Exponentially growing cells are treated with the inhibitor. *II*, DNA is cross-linked with trioxsalen *in vivo*. *III*, Cells are cultured with [^{3}H]thymidine in the presence of the drug to label the newly initiated DNA chains. *IV*, DNA is isolated and subjected to electrophoresis under denaturing conditions. During electrophoresis cross-linked genomic DNA forms a slow-moving peak, whereas the nascent DNA fragments synthesized at origins of replication form a fast-moving peak. The radioactivity included in the fast-moving peak is directly proportional to the rate of initiation of DNA synthesis.

55°C for 1 h and was electrophoresed in 1% agarose gel prepared and run in 10 mM phosphate buffer, pH 7 (12). The amount of DNA loaded onto the gel was determined by reading the ^{14}C counts of the samples. To follow the distribution of the radioactivity in the gel, the respective lanes were cut into 1-cm bands, dissolved in 0.5 ml of 0.1 M HCl by a brief boiling and counted.

RESULTS AND DISCUSSION

To discriminate between inhibitors of initiation and elongation of DNA synthesis, one has to assess the exact contribution of these two components in the overall DNA synthesis. There are few reports in which the initiation and elongation of DNA were studied separately. Years ago several articles from Huberman's (13) laboratory approached this problem using fiber autoradiography. Although fiber autoradiography is the most direct way to measure both the numbers of initiation events and the rates of chain elongation, the method registers individual replication events and one needs several experiments to obtain statistically significant results. Also, it routinely takes about 1 month to develop the autoradiographs. Alternatively, Painter (14) used alkaline sucrose gradient sedimentation to assess the initiation rates of DNA synthesis in norma and after X-irradiation. Although easy to perform, this method is not very sensitive and is difficult to quantify. Recently, a method was developed that was a combination of isotopic labeling protocol and a two-dimensional gel electrophoresis replicon mapping procedure (6). However, the method is applicable only to individual replicons for which molecular probes are available and this limits its application. In addition, it is rather time consuming and technically difficult to perform. We have developed an alternative protocol for evaluation of the effect of inhibitors of DNA synthesis on the level of replicon initiation in mammalian cells. Briefly, exponentially growing cells are treated with the inhibitor and DNA is cross-linked with trioxsalen. The latter is a nonionic psoralen derivative that can penetrate the cell membranes, intercalates into DNA, and after photoreactivation, forms covalent interstrand cross-links. The cross-linked cells are incubated with [^{3}H]thymidine in the presence of the drug. During the incubation, [^{3}H]thymidine will be incorporated into three classes of newly synthesized DNA: (*a*) Repair will be taking place and as a result, 20–30 nucleotide long stretches of labeled DNA will be synthesized (15). They will be covalently attached by both their ends to the high molecular weight DNA and during the subsequent procedures will remain bound to it (16). (*b*) Elongation of the already initiated DNA chains will continue during the first few minutes of labeling until the replication forks reach trioxsalen cross-links, where they will stall. This will produce DNA stretches covalently linked by their 5′ ends to the high molecular weight DNA and during the subsequent procedures will remain bound to it. (*c*) Finally, short DNA fragments initiated at origins of replication, located between trioxsalen cross-links, will be synthesized. They will not be ligated to the high molecular weight DNA and under denaturing conditions will be released from bulk DNA. Upon electrophoresis under denaturing conditions, cross-linked genomic DNA will form a slow-moving peak, whereas the fragments synthesized at origins of replication will form a fast-moving peak (Fig. 1). Provided equal amounts of DNA were loaded onto the gel, the amount of ^{3}H counts incorporated in the low molecular mass peak containing the short nascent DNA fragments would be directly and quantitatively proportional to the number of initiation events and could be used as a measure for the rate of replicon initiation. It should be noted that here we operationally use the term "initiation" to describe all steps leading to the synthesis of short nascent DNA chains and not any of the specific events such as the assembly of the initiation complex, the unwinding of the origin sequences, etc.

In the present study, we applied this technique to study the effect of the amino acid mimosine on DNA synthesis. We performed two types of experiments. First, we compared the rates of DNA synthesis and the rates of replicon initiation in exponentially growing F_4N cells incubated for 1 h with increasing concentrations of mimosine. These data are presented in Fig. 2. It can be seen that at all mimosine

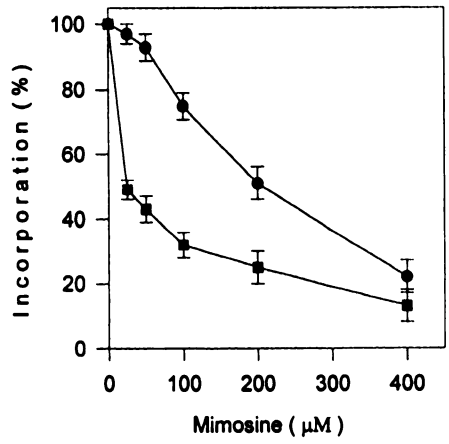

Fig. 2. Inhibition of genomic DNA synthesis (●) and replicon initiation (■) by different doses of mimosine. To determine the rate of genomic DNA synthesis, exponentially growing F_4N cells prelabeled uniformly with [^{14}C]thymidine were treated with 25–400 μM mimosine for 1 h and labeled with [^{3}H]thymidine for 30 min in the presence of the drug. Genomic DNA was isolated and its specific radioactivity was determined. The specific radioactivity of DNA in percentage of that of the untreated with the inhibitor control cells was plotted *versus* dose. To determine the replicon initiation rates, aliquots of the mimosine-treated F_4N cells were cross-linked with trioxsalen and labeled with [^{3}H]thymidine for 45 min in the presence of the inhibitor. DNA was isolated and subjected to electrophoresis under denaturing conditions. The amount of radioactivity in the fast-moving peak was presented as percentage of that of the untreated with the inhibitor control cells and plotted *versus* dose. Each point is a mean of three experiments. *Bars*, SD.

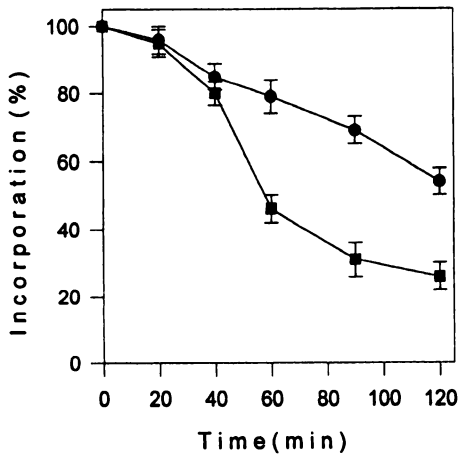

Fig. 3. Inhibition of genomic DNA synthesis (●) and replicon initiation (■) after treatment with 200 μM mimosine for different time intervals. Exponentially growing F_4N cells were treated with 200 μM mimosine, and at different time intervals cells were labeled with [^{3}H]thymidine for 30 min in the presence of the inhibitor. The specific radioactivity of genomic DNA in percentage of the control is plotted *versus* time. To determine the replicon initiation rates, aliquots of the mimosine-treated cells were cross-linked with trioxsalen and labeled with [^{3}H]thymidine for 45 min in the presence of the drug. DNA was isolated and subjected to electrophoresis under denaturing conditions. The amount of radioactivity found in the fast-moving peak in percentage of the control was plotted against time. Each point is a mean of three experiments. *Bars*, SD.

concentrations initiation was inhibited to a higher extent than the overall DNA synthesis, the differences being more pronounced at lower doses (up to 200 μM) of the drug. It should be noted that at the lowest doses (25 and 50 μM), while the overall DNA synthesis was inhibited only less than 10%, the inhibition of replicon initiation was more than 50%. In the second type of experiment, the cells were incubated with 200 μM mimosine for different periods of time (Fig. 3). It can be seen that after a lag period of about 30 min, the rate of [^{3}H]thymidine uptake began to decline and the decline of the rate of initiation events was steeper than the decline of the rate of the overall DNA synthesis. Between the first and second h of incubation, the rate of replicon initiation was about 40% lower than the rate of the overall DNA synthesis.

Furthermore, we compared the effect of mimosine on replicon initiation and genomic DNA synthesis with those of hydroxyurea and aphidicolin. The two inhibitors are known to inhibit the elongation step of DNA synthesis, although the mechanism of their action is different. Hydroxyurea affects the levels of the cellular deoxyribonucleotide triphosphate pools (17) and aphidicolin is an inhibitor of DNA polymerases α, δ, and ε (reviewed in Ref. 18). The results are presented in Figs. 4 and 5. It can be seen that both agents inhibited the overall DNA synthesis to a greater extent than replicon initiation. These results confirm the findings that inhibition of initiation of DNA synthesis is not the primary effect of hydroxyurea and aphidicolin and that the main target of these drugs is the elongation of the already initiated DNA chains. The results also show that the ratio of the rates of initiation and overall DNA synthesis is affected by hydroxyurea and aphidicolin in a manner opposite to that of mimosine (Fig. 3). The data reveal that mimosine affects replication not only through its action on the deoxyribonucleotide metabolism as proposed by Gilbert *et al.* (7). If the response to mimosine of the two steps of DNA synthesis, *i.e.*, initiation and elongation, reflected only their sensitivity to nucleotide triphosphate concentration, the inhibition of the overall DNA synthesis would have been faster than the inhibition of initiation because DNA elongation is the major synthetic event. It consumes more deoxyribonucleotide triphosphates and would be more dependent on deoxyribonucleotide concentrations than initiation, and, consequently, elongation would be more affected as was the case with hydroxyurea. In the presence of 5 mM hydroxyurea, the elongation step was rapidly blocked by the lack of deoxyribonucleotide triphosphates, whereas initiation was less affected and as a result short nascent DNA fragments were synthesized at a higher rate than the overall DNA synthesis: at 30 min after introduction of hydroxyurea, initiation proceeded with about 80% of the control rate, whereas the overall DNA synthesis rate was decreased to about 45% of the control rate (Fig. 4). The fact that unlike inhibitors of elongation, mimosine inhibited replicon initiation to a much greater extent than the overall DNA synthesis suggested that the drug exerted its inhibitory effect on DNA synthesis by a mechanism including inhibition of replicon initiation.

To check this suggestion, we compared the dose-response curves for the effect of mimosine on DNA synthesis and on replicon initiation (Fig. 2) with those for low doses of γ-irradiation (Fig. 6), an agent known to exert its inhibitory effect on DNA synthesis by slowing down initiation of replication (14, 19, 20). It can be seen that the relationship between the rates of incorporation of [^{3}H]thymidine into genomic DNA and into the short nascent DNA chains is similar for γ-irradiation and mimosine. At every dose of the agents, the inhibition of replicon initiation was greater than the inhibition of the overall DNA synthesis and this shows that mimosine does act like an inhibitor of initiation of DNA replication.

Our data support the suggestion that mimosine may be a specific

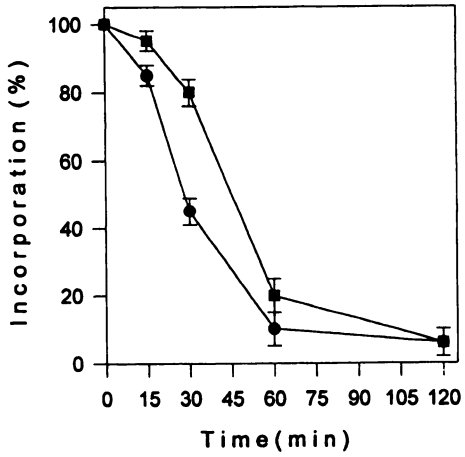

Fig. 4. Inhibition of genomic DNA synthesis (●) and replicon initiation (■) after treatment with 5 mM hydroxyurea for different time intervals. Determination of the rates of genomic DNA synthesis and replicon initiation were carried out as in Fig. 3.

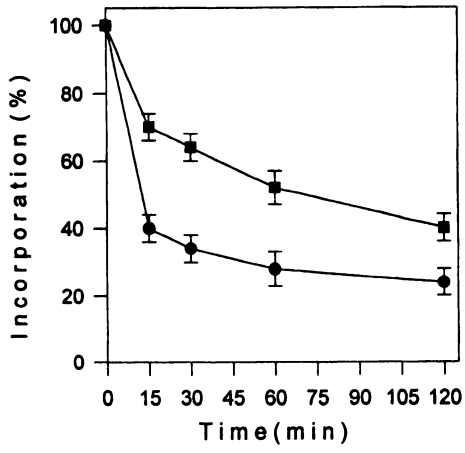

Fig. 5. Inhibition of genomic DNA synthesis (●) and replicon initiation (■) after treatment with 0.6 μM aphidicolin for different time intervals. Determination of the rates of genomic DNA synthesis and replicon initiation were carried out as in Fig. 3.

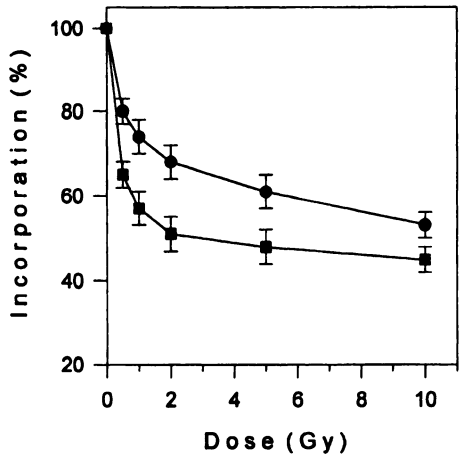

Fig. 6. Effect of γ-radiation on genomic DNA synthesis (●) and replicon initiation (■). Exponentially growing F_4N cells received 0.5–10 Gy of γ-radiation. Determination of genomic DNA synthesis and replicon initiation rates were carried out as in Fig. 2.

inhibitor of replicon initiation (4–6). At present, it is not clear by what mechanism mimosine inhibits initiation at replication origins. It has been reported that mimosine has a wide range of effects on the cell. Thus mimosine is a strong iron-chelating agent (7, 21, 22). *In vitro* it inhibits various mammalian enzymes such as ribonucleotide reductase (21), tyrosinase and dopamine β-hydroxylase (23), deoxyhypusyl hydroxylase (24), and histone H1 kinase (25). It also should be kept in mind that in high concentration mimosine could disrupt chromatin structure (26, 27). It could be speculated that replication origin regions being with lower thermodynamic stability (reviewed in Ref. 28) may be more susceptible to its action. Interestingly, mimosine was shown to increase both $p21^{WAF1/CIP1/SDI1}$ protein levels and p21 mRNA and indirectly inhibited cyclin E-associated kinase activity, without affecting its cytoplasmic concentration (2). These data suggest that the effect of mimosine is very complex, may have more than one target, and it is difficult to separate primary from downstream effects.

The interest toward chemicals that affect DNA synthesis and can be used in chemotherapy in the treatment of cancer is explicably great, and it is important to know what the mechanism of their action on DNA replication is. The protocol we have developed for determination of the effect of replication inhibitors on the initiation of DNA synthesis has some well-defined advantages over other protocols currently in use. It is less time consuming and is easy to be quantified and reproduced. It also does not require any additional conditions such as prior knowledge of DNA primary structure or availability of molecular probes. That is why the method can be a useful tool for the elucidation of the mechanism of action of drugs that affect DNA synthesis.

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