Mimosine, a novel inhibitor of DNA replication, binds to a 50 kDa protein in Chinese hamster cells

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ABSTRACT

We recently demonstrated that the plant amino acid, mimosine, is an extremely efficacious inhibitor of DNA replication in mammalian cells [P. A. Dijkwel and J. L. Hamlin (1992) *Mol. Cell. Biol.* 12, 3715-3722; P. J. Mosca *et al.* (1992) *Mol. Cell. Biol.* 12, 4375-4383]. Several of its properties further suggested that mimosine might target initiation at origins of replication, which would make it a unique and very useful inhibitor for studying the regulation of DNA synthesis. However, mimosine is known to chelate iron, a cofactor for ribonucleotide reductase. Thus, the possibility arose that mimosine functions *in vivo* simply by lowering intracellular deoxyribonucleotide pools. In the present study, we show that, in fact, it is possible to override mimosine inhibition *in vivo* by adding excess iron; however, copper, which is not a substitute for iron in ribonucleotide reductase, is equally effective. Evidence is presented that mimosine functions instead by binding to an intracellular protein. We show that radiolabeled mimosine can be specifically cross-linked to a 50 kDa polypeptide (termed p50) *in vitro*. Binding to p50 is virtually undetectable in CHO cells selected for resistance to 1 mM mimosine, arguing that p50 is the biologically relevant target. p50 is not associated with the cellular membrane fraction and, hence, is probably not a channel protein. Furthermore, the binding activity does not vary markedly as a function of cell cycle position, arguing that p50 is not a cyclin. Finally, both iron and copper are able to reverse the mimosine-p50 interaction *in vitro*, probably explaining why both metal ions are able to overcome mimosine's inhibitory effect on DNA synthesis *in vivo*.

INTRODUCTION

We are attempting to identify and characterize the two components that are required for initiation of replication in mammalian chromosomes, namely, origins of replication and *trans*-acting initiator proteins. As a model system, we are studying the amplified dihydrofolate reductase (DHFR) domain in the metho-

trexate-resistant Chinese hamster ovary cell line, CHO 400 (1). Several different experimental approaches suggest the presence of an origin downstream from the DHFR gene (2-8). Unfortunately, cloned sequences from this region do not behave as origins when tested for their ability to replicate autonomously *in vivo* or *in vitro* (3,9; J. L. Hamlin, unpublished observations). Thus, the exact sequence elements required for origin function have not yet been identified. As a consequence, it also has not been possible to identify potential initiator proteins by searching for sequence-specific DNA binding proteins.

We recently presented evidence that the plant amino acid, mimosine, may specifically inhibit initiation at mammalian origins of replication (10). This compound could therefore be an extremely useful agent for identifying proteins involved in the initiation reaction.

When mimosine is used to collect CHO 400 cells near the G₁/S boundary after release from a G₀ block, replication intermediates in the DHFR initiation locus are undetectable by a two-dimensional gel replicon mapping technique (10-12), suggesting that mimosine effectively prevents replication fork set-up. Furthermore, the initial rate of uptake of ³H-thymidine into DNA extrapolates to zero regardless of the length of the mimosine block, arguing that initiation of new replicons does not occur in the presence of mimosine (11).

In addition, when added to an *asynchronous* population of CHO 400 cells, mimosine inhibits ³H-thymidine incorporation, but complete inhibition requires ~2.5 h (10). This slow-stop phenotype is reminiscent of the behavior of temperature-sensitive initiation mutants in *E. coli* in response to a shift from permissive to non-permissive temperatures (13). When mimosine is added to log cells and the behavior of replication intermediates is examined on 2-D gels, further initiations in the amplified DHFR locus are prevented after ~1 h, but replication forks in operation at the time of drug addition appeared to continue on to their destinations (10).

Therefore, mimosine behaves very differently than either aphidicolin or hydroxyurea (10-12,14), inhibitors of DNA polymerase (15) and ribonucleotide reductase (16), respectively: both agents allow significant escape synthesis to occur when used to synchronize cells near the G₁/S boundary (11,12,14), and both inhibit ³H-thymidine uptake into DNA almost immediately when added to log cells (10,12). Based on its unique properties, we have suggested that mimosine might inhibit either an origin-binding

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protein or some other protein required to establish replication forks during the initiation reaction (10,12).

However, several other observations have cast some doubt on this model. We have recently shown that mimosine-arrested cells are no longer sensitive to the acute down-regulation of initiation that occurs in response to ionizing radiation (17), suggesting that the mimosine-sensitive step is beyond this radiation-sensitive regulatory step. In addition, when asynchronous CHO-K1 cells are treated with mimosine, more than 4 h is required to completely clear replication forks from the DHFR locus, whereas uptake of ^3H -thymidine into DNA is almost completely inhibited by 2.5 h (10,12). We have also shown that SV40 replication intermediates are chased more slowly into completed viral genomes in the presence of mimosine (R. J. Kalejta and J. L. Hamlin, submitted for publication). These observations raise the possibility that mimosine might slow the rate of chain elongation in addition to, or instead of, inhibiting initiation *per se*.

Several other pieces of information raise the possibility that mimosine might inhibit replication *in vivo* by affecting the activity of ribonucleotide reductase: 1) mimosine is known to chelate iron (18–20); 2) ribonucleotide reductase is an iron-dependent enzyme (reviewed in 16); and 3) iron chelators (including those in the mimosine family) inhibit ribonucleotide reductase *in vitro* (21,22).

In this report, we show that, in fact, iron can reverse the inhibitory effects of mimosine on DNA replication when added to CHO cells *in vivo*. However, copper, which is not known to substitute for iron in ribonucleotide reductase (16), is similarly effective. Thus, it cannot be concluded from this approach that mimosine functions by removing iron from ribonucleotide reductase. Moreover, we show that mimosine can be cross-linked both *in vivo* and *in vitro* to a 50 kDa polypeptide (p50), and that cross-linking can be prevented by addition of iron or copper. Evidence is also presented that p50 is the biologically relevant target. These data are not consistent with the proposal that mimosine functions solely as a chelating agent. We also define several of the properties of p50, which will be important for its subsequent purification and characterization.

MATERIALS AND METHODS

Cell culture and synchronization

A clonal isolate of CHO-K1 cells with a doubling time of 12–14 h was maintained in Minimal Essential Medium (MEM) supplemented with non-essential amino acids (GIBCO) and 10% Bovine Serum Product (HyClone) in an atmosphere of 5% CO_2 . Cells were grown on 10 or 15 cm plates, or in 24-well cluster dishes as required.

For the cell cycle experiment, cultures were arrested in G_0 by incubation in isoleucine-free medium for 24 h and were then released into complete medium containing mimosine (Aldrich Chem.) for 14 h. The cells were washed once with warm, serum-free medium and were returned to drug-free complete medium. Fluorescence-activated cell sorter (FACS) analysis was performed for each time point at which samples were prepared for cross-linking (10).

A population of cells resistant to 1 mM mimosine was developed by subjecting CHO-K1 cells to seven increments in mimosine concentration over the period of 15 months, beginning at 100 μM (25).

Effects of metal salts on the inhibition of DNA replication by mimosine

CHO-K1 cells were plated 24 h before use at a density of 10^5 cells/well in 24-well cluster dishes. The cultures then received 200 μM mimosine, alone or in combination with the indicated concentrations of FeCl_3 or CuSO_4 . In some experiments, the metal salts were added after a 3 h incubation period with mimosine. DNA synthesis was monitored by labeling cells for 30 or 60 min with 2 $\mu\text{Ci}/\text{ml}$ ^3H -thymidine (80 Ci/mM; Dupont/NEN) + 0.2 $\mu\text{g}/\text{ml}$ unlabeled thymidine. Label incorporated into DNA was then determined as described previously (10). Data were plotted using the Sigmaplot program (version 4.1).

Preparation of lysates and extracts

CHO-K1 cells or their mimosine-resistant derivatives were grown to confluence, washed once with phosphate-buffered saline, and drained almost to dryness. All subsequent manipulations were performed at $\sim 25^\circ\text{C}$ unless otherwise noted. Lysates were prepared by adding 40 or 80 μl of lysis buffer to each 10 or 15 cm plate, respectively, scraping the cells with a plastic policeman, transferring to a microfuge tube, and gently triturating the suspension with a pipette tip. Lysis buffer contained: 20 mM Hepes, pH 7.6, 3 mM MgCl_2 , 20 mM NH_4Cl , 2% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-tosyl-L-lysyl-chloromethyl ketone, 25 $\mu\text{g}/\text{ml}$ leupeptin and 25 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor. After incubation at 37°C for 30 min with ^3H -mimosine (unless specified otherwise), the lysates were photo-crosslinked as described below. The final extracts represented $\sim 2 \times 10^8$ cells/ml.

Cell fractionation

Cell fractionations were performed by a slight modification of two published methods (23,24). All preparations were carried out at 4°C . A 15 cm plate of asynchronous CHO-K1 cells was harvested with a plastic policeman and resuspended in 0.2 ml lysis buffer without Triton X-100. The cell suspension was homogenized with a tight-fitting Dounce homogenizer until 95% of nuclei could be stained with Trypan blue. The resulting homogenate was passed through a 21.5 gauge needle three times and was then centrifuged at $\sim 5000 \times g$ for 5 min to yield soluble supernatant and nuclear pellet fractions. The supernatant fraction was collected and re-centrifuged at $150\,000 \times g$ for 30 min. The resulting supernatant was referred to as the cytoplasmic fraction and the pellet as the microsomal or vesicle fraction. The nuclear pellet was washed once with lysis buffer lacking Triton X-100 and was then treated with lysis buffer containing 2% Triton X-100 for 5 min. After vortexing, the nuclear lysate was separated into nuclear soluble and insoluble fractions by centrifugation at $\sim 5000 \times g$ for 5 min. All of the resulting fractions were suspended in the same final volume to perform the cross-linking experiments described below.

Photochemical cross-linking

Lysate or cell fractions (80–100 μl) were mixed with 80 μCi ^3H -mimosine (7.4 Ci/mmol; Amersham Radiochemical Corp.; final concentration $\sim 100 \mu\text{M}$) and were incubated for 30 min at 37°C . Each sample was then placed in one well of a 48-well cluster dish (Costar) on ice and was immediately illuminated for 15 s (unless otherwise noted) at a distance of ~ 10 cm with a

Figure 1. Both iron and copper can reverse the effect of mimosine on DNA synthesis. (A) Asynchronous CHO-K1 cells were incubated either with a mixture of 200 μ M mimosine plus the indicated concentrations of FeCl_3 for 3 h (open bars), or were preincubated with 200 μ M mimosine for 3 h before adding iron for an additional 3 h (filled bars). Cells were then labeled for 30 min with ^3H -thymidine in the presence of the drug and heavy metals. The total d.p.m. incorporated into the control samples was 5700 ± 300 . (B) Same as panel A, except that copper was used in place of iron and labeling with ^3H -thymidine was for 60 min. The total d.p.m. incorporated into the control samples was 7400 ± 400 . (C) Asynchronous CHO-K1 cells were incubated with the indicated concentrations of iron (open bars) or copper (filled bars) for 3 h and were then labeled with ^3H -thymidine for 60 min. The total d.p.m. incorporated into the control samples was 8000 ± 500 . Data are expressed as a percentage of controls not receiving additives. Error bars indicate the standard deviation of duplicate samples.

broad-spectrum xenon lamp (ILC model LX300UV) at a setting of 20 amp (ILC Illuminator power supply). Concentrated polyacrylamide gel electrophoresis sample buffer was added, and samples were boiled for ~5 min and clarified in a microcentrifuge for 2–5 min prior to separation on a 10% gel. When samples were compared on a single gel, each well contained proteins from an equal number of cells (~2×10⁶/well). The resulting gels were stained with Coomassie Brilliant Blue, photographed, treated with Entensify fluorographic enhancer (DuPont/NEN) and dried. The dried gels were exposed to Kodak X-AR film using an intensifying screen at -70°C.

RESULTS

Inhibition of DNA replication by mimosine can be reversed with metal salts

Mimosine is known to chelate iron, copper and zinc, as well as other metals (18–20,26). It is possible that mimosine inhibits DNA replication by removing iron from ribonucleotide reductase, thereby lowering deoxyribonucleotide pool levels. We therefore determined whether mimosine's effect on replication could be overcome with excess iron.

In the first experiment, asynchronous CHO-K1 cells were treated for 3 h either with 200 μ M mimosine or with 200 μ M mimosine plus different concentrations of FeCl_3 . Alternatively, cells were pretreated with mimosine for 3 h, FeCl_3 was added, and incubation was continued for an additional 3 h. Replicating DNA was then labeled with ^3H -thymidine in the presence of the various additives for 1 h. The amount of ^3H -thymidine incorporated into DNA was expressed as a percent of a control culture containing no additives. In Figure 1A (open bars), a 3 h incubation with 200 μ M mimosine alone depresses the rate of DNA replication to background levels, but this inhibition can be completely overcome by co-incubating with FeCl_3 at concentrations of 100 μ M or greater. This result would seem to support the contention that mimosine inhibits DNA replication by depriving ribonucleotide reductase of required iron.

However, as shown in Figure 1B, copper is also able to override the inhibitory effect of mimosine on DNA replication in CHO-K1 cells (open bars), even though copper is not known to substitute for iron in ribonucleotide reductase (16). Note that it takes four to five times more copper than iron (~300 and ~75 μM , respectively) to restore the replication rate to ~50% of control values. As we will discuss below, this could relate to the fact that the saturating ratios of the mimosine family to iron and copper are substantially different [3:1 (22) and 1:2 (26), respectively].

Virtually the same results were obtained when cells were pretreated with mimosine alone for 3 h prior to the 3 h incubation with mimosine plus iron or copper (Fig. 1A and B, solid bars). The data in Figure 1C additionally show that neither iron nor copper by itself affects DNA replication at concentrations as high as 200 and 400 μM , respectively. However, 800 μM copper appears to be toxic, probably explaining why 800 μM copper does not appear to reverse the effects of mimosine in the experiment shown in Figure 1B.

Since both iron and copper can restore the rate of DNA replication in mimosine-treated cells to control levels, it is not possible to conclude from this experiment that mimosine functions by chelating the iron required by ribonucleotide reductase (Fig. 2A). In fact, these data cannot rule out a second model in which mimosine inhibits replication by binding to and inhibiting an intracellular protein (Fig. 2B). Iron or copper could then bind to mimosine, inhibiting its ability to bind to the intracellular target. In other words, the excess iron or copper might inactivate mimosine, as opposed to re-activating an iron-deprived target protein.

Mimosine can be photo-crosslinked directly to a 50 kDa protein in CHO cells

To address this alternative possibility, we asked whether mimosine binds to a cellular protein. In pilot experiments, we attempted to identify a binding protein by several classic approaches, including affinity chromatography and filter-binding assays. In all cases, we were unable to detect any mimosine binding activity in CHO cell lysates (P. J. Mosca, unpublished observations).

Figure 2. Models for mimosine inhibition and reversal by iron. In (A) mimosine robs the iron from the active target protein (TP_A) by chelation, rendering it inactive (TP_I); added iron then over-titrates the ambient mimosine and resupplies iron to the target protein. In this scenario, mimosine does not necessarily associate with the target directly. In (B) mimosine binds directly to the active target protein and renders it inactive; excess iron (or copper) then simply competes more effectively than the target protein for mimosine.

Since the inhibitor is maximally effective only at concentrations above $\sim 100 \mu\text{M}$ (P. J. Mosca, unpublished observations), its interaction with the target could have an unfavorably high off-constant. A cross-linking strategy was therefore developed to prevent the drug from dissociating from its target(s). This protocol takes advantage of the fact that mimosine absorbs strongly in the ultraviolet range ($\sim 1.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at $\sim 283 \text{ nm}$; data not shown).

Cell lysates were prepared and preincubated with ^3H -mimosine for 30 min at 37°C , and were then illuminated with a xenon lamp for various time intervals (37°C was chosen to mimic the condition under which mimosine exerts its effect *in vivo*). The extracts were separated on a polyacrylamide gel, which was then stained and fluorographed as described in Materials and Methods.

As shown in Figure 3A, the spectrum of Coomassie-stained polypeptides is not dramatically affected by irradiation, although an increasing amount of aggregated material collects at the stacking/separating gel interface. Presumably, this results from non-specific cross-linking of cellular proteins.

As shown by the autoradiograph of the same gel in Figure 3B, binding of ^3H -mimosine to cellular proteins cannot be detected in the unirradiated control extract. However, illumination for times as short as 5 s results in the appearance of a 50 kDa labeled polypeptide, as well as significant quantities of labeled material at the top of the gel. With longer irradiation times, both the 50 kDa species and the high molecular weight material increase in intensity, although background binding becomes significant at 30 s and longer.

Figure 3. ^3H -mimosine can be directly cross-linked to a 50 kDa protein in CHO cell lysates. Clarified CHO cell lysates were incubated with $100 \mu\text{M}$ ^3H -mimosine for 30 min and were illuminated on ice with a Xenon lamp for 0, 5, 15, 30 or 45 s, as described in Materials and Methods. Protein from each sample ($\sim 2 \times 10^6$ cell equivalents per sample) were separated on an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue (A). The gel was then treated with fluorophor, dried, and autoradiographed (B).

Binding to the 50 kDa protein is dramatically reduced in mimosine-resistant CHO cells

Although the binding of ^3H -mimosine to p50 *in vitro* appears to be relatively specific at moderate irradiation times, it cannot be concluded that this binding activity represents the bona fide intracellular target for mimosine. To address this point directly, CHO-K1 cells were subjected to increasing concentrations of mimosine in an attempt to obtain a resistant variant that had either amplified the gene that encodes p50 or that had lost the p50 mimosine-binding activity by mutation. After seven incremental increases in drug concentration over the period of 15 months, a cell population was obtained that is resistant to 1 mM mimosine (~ 10 -fold higher than the lethal dose for CHO-K1 cells; unpublished observations). Extracts were prepared from this resistant population and were irradiated in the presence of ^3H -mimosine to detect binding activity.

As shown in Figure 4B, these cells appear to have become resistant by loss of p50 mimosine-binding activity, since the labeled 50 kDa band is barely detectable in extracts from resistant cells (R), even though the Coomassie Blue-stained gel clearly shows that approximately the same amount of protein is present in each sample (compare to the control extract from mimosine-sensitive CHO-K1 cells; K1 in the figure). In very long film exposures, a faint band can be detected in extracts from resistant

Figure 4. The 50 kDa mimosine binding activity is not detected in extracts from mimosine-resistant CHO cells. One confluent 10 cm plate of parental CHO-K1 cells (K1) or of mimosine-resistant cells (R) growing in mimosine-free medium was lysed in 50 μ l of lysis buffer, resulting in 80–85 μ l of lysate ($\sim 2 \times 10^6$ cell equivalents per sample). ^3H -mimosine was added to each sample (to $\sim 100 \mu\text{M}$) in adjacent wells of a 48-well cluster dish. After a 30 min incubation at 37°C , the samples were illuminated on ice for 15 s. Samples were then treated as in Figure 3. The Coomassie-stained gel is shown in (A) and the fluorograph in (B). The sizes in kDa of radioactive markers (m) are indicated.

cells, which represents less than 10% of the wild-type binding activity (data not shown).

p50 is probably not a transporter

While decreased binding to p50 appears to be responsible for resistance to mimosine, p50 could represent a transporter that has lost its ability to carry mimosine into the cell, rather than the bona fide target protein. Even though the extracts used in the experiments in Figures 3 and 4 contain $\sim 1\%$ Triton X-100 (which should effectively solubilize membrane components), it is conceivable that a proposed transporter could still bind efficiently to mimosine under these conditions. We addressed this possibility in two ways.

In the first approach, we determined the intracellular distribution of p50 in drug-sensitive CHO-K1 cells. Cells were homogenized and cytoplasmic, vesicle, nuclear pellet, and soluble nuclear protein fractions were prepared by standard procedures (23,24, and Materials and Methods). Each fraction was irradiated in the presence of ^3H -mimosine, and samples from equal numbers of cells were then separated on a polyacrylamide gel, stained with Coomassie Brilliant Blue, and fluorographed. The staining patterns in Figure 5A indicate that the four cellular fractions

Figure 5. Cellular distribution of p50. Cell fractionation and cross-linking were performed as described in Materials and Methods. Samples containing protein from 5×10^6 cell equivalents were loaded into each well. The Coomassie-stained gel and the autoradiograph are shown in (A) and (B), respectively. Cyt—cytoplasmic extract; NE—nuclear extract; NP—nuclear pellet; Ves—vesicle fraction; m—molecular weight markers.

contain markedly different and largely non-overlapping distributions of proteins. The fluorograph shown in Figure 5B indicates that p50 is associated primarily with the soluble cytoplasmic and nuclear fractions (although a small but variable amount can usually be detected in the two other compartments).

In a second approach, the rates of ^3H -mimosine uptake were determined in CHO-K1 cells and on several independent clones isolated from the mimosine-resistant population. The initial transport rates (measured during the first 3 min after addition of ^3H -mimosine to individual resistant clones and calculated on a per cell basis) was depressed as much as 50% relative to drug-sensitive CHO-K1 cells (H.-B. Lin, manuscript in preparation). However, the growth rate of these resistant clones is also about half that of parental CHO-K1 cells, suggesting that reduced transport could be coupled to metabolic rate. We therefore consider it unlikely that transport differences of this magnitude could explain the virtual abolition of the 50 kDa mimosine binding activity in mimosine-resistant cells (Fig. 4B). In combination with results on cellular distribution (Fig. 5B), these data argue that p50 is probably not a transporter for mimosine.

The level of p50 does not vary dramatically as a function of cell cycle position

We have shown previously that mimosine inhibits DNA replication at the beginning of the S period when delivered to cells during G_1 (10,11) and when administered to cells in the first two-thirds of the S phase (10). It is therefore conceivable that mimosine inhibits a cyclin or other protein that functions during these intervals (see ref. 27 for review). To characterize mimosine-binding activity as a function of cell cycle position, CHO-K1 cells were blocked in G_0 by isoleucine starvation and were then released from this block into mimosine. By 14 h, at which time

Figure 6. The 50 kDa mimosine-binding activity as a function of cell cycle position. Cells were synchronized as described in Materials and Methods, and samples were harvested at the indicated times and cross-linked to ^3H -mimosine. The protein from equivalent numbers of cells was loaded into each well. (A) Coomassie-stained gel. (B) Fluorograph.

the population has arrived at the G_1/S boundary, mimosine was removed and cells were allowed to enter and traverse the 7 h S period. Samples were taken at various time intervals beginning at G_0 , and FACS analyses were performed to ascertain cell cycle position (data not shown). Total cell extracts were prepared and ^3H -mimosine binding activity was determined.

The autoradiograms in Figure 6B show that p50 binding activity is present during every cell cycle interval examined in this study, including during G_0 . Therefore, even though mimosine appears to have no effect on cells during G_1 , G_2 or mitosis (10), the 50 kDa mimosine-binding protein is nevertheless present. This experiment does not rule out the possibility that post-translational modification affects the activity of the target protein without affecting its ability to bind mimosine.

Both iron and copper can prevent the binding of ^3H -mimosine to p50

The fact that we were able to identify a mimosine-binding protein suggests that mimosine exerts its effect by the direct binding mode pictured in Figure 2B. Iron and copper might then be able to override the inhibitory effect of mimosine on DNA replication by binding to the drug and preventing it from binding to p50. To test this notion directly, mimosine was added to extracts from CHO-K1 cells and the extracts were irradiated either in the absence or the presence of iron or copper. As shown in Figure 7, both metals largely prevent cross-linking of ^3H -mimosine to p50 at concentrations similar (but not identical) to those that override mimosine inhibition *in vivo*. Thus, iron and copper appear to inactivate the inhibitor itself, as proposed in the model in Figure 2B. This, of course, does not rule out the possibility that chelation of iron by mimosine might also inhibit ribonucleotide reductase (see Discussion).

DISCUSSION

Mimosine is a tyrosine analogue that is manufactured by *Leucaena leucocephala* and *Mimosa pudica* (19,28). These plants were potential sources of livestock feed in Australia, but were found to be relatively toxic and to induce hair loss in sheep (29–31). The toxic agent was found to be mimosine (30), which was then investigated for possible use as a defleecing agent (31). Since then, mimosine has been shown to inhibit several mammalian enzymes *in vitro* that are presumably unrelated to DNA replication, including tyrosinase (32), dopamine β -hydroxylase (32), L-dopa decarboxylase (33) and alkaline phosphatase (34).

In spite of these activities, however, when mimosine is added to an asynchronous culture of mammalian cells, it appears to affect only those cells that either are in the S period or that are about to enter S (10). Indeed, mimosine is the most efficacious inhibitor of DNA replication that we have ever encountered: it completely prevents replication fork set-up in the CHO DHFR domain at the beginning of S (10–12) and, unlike aphidicolin (11,15,35) or hydroxyurea (12,14), it effectively prevents further DNA synthesis for periods as long as 48 h (10).

Mimosine is known to chelate iron and other metals (e.g., 18–20), and therefore could exert its effects on replication by indirectly inhibiting ribonucleotide reductase or other metal-requiring enzymes. In fact, we were able to overcome mimosine inhibition with added iron. However, copper was equally effective, and copper is not known to substitute for iron in ribonucleotide reductase.

Thus, even though this 'add-back' approach has been used to attempt to show that mimosine and other inhibitors exert their effects by chelation (e.g., 22), we suggest that it cannot distinguish between the two possibilities for mimosine action

Figure 7. Both iron and copper prevent the binding of ^3H -mimosine to p50. CHO-K1 cell lysates were incubated with ^3H -mimosine (100 μM) at 37°C for 30 min. FeCl_2 and CuSO_4 were added at the indicated concentrations and incubation was continued for an additional 15 min. The samples were then irradiated and binding to p50 was assessed as described in Materials and Methods.

outlined in Figure 2, i.e., (i) leeching of required iron from the target enzyme, which can be overcome with excess iron, or (ii) binding to a cellular target protein, which can be prevented by inactivating mimosine with metals. Furthermore, it is not possible to conclude that a compound inhibits an enzyme involved in nucleotide metabolism based solely on the lowering of pool levels, since such an effect could result indirectly from an inhibition of DNA synthesis by another route.

Our second approach to identifying mimosine's intracellular target suggests, in fact, that mimosine has a different (or an additional) effect. A 50 kDa mimosine-binding polypeptide was detected by cross-linking ^3H -mimosine either *in vivo* (25) or *in vitro* (this report). Since mimosine binding activity (but not necessarily the protein itself) is absent in cells made resistant to the drug, we are reasonably confident that p50 represents a biologically significant target. However, to formally prove this suggestion, it will be necessary to clone the wild-type and resistant p50 cDNAs and to use them to attempt to confer drug-sensitivity or -resistance to resistant and sensitive CHO cells, respectively. In addition, until we are able to obtain antibodies to p50, we will not be able to determine whether mimosine-resistant cells lack the p50 protein or only its binding activity.

The fact that iron and copper can largely prevent the binding of mimosine to p50 lends weight to the argument that p50 is a significant target for mimosine. However, one aspect of this result deserves comment. Were p50 to be the only intracellular mimosine target, then one would expect that the concentration of

iron and copper required to override mimosine's effect on DNA replication or its binding to p50 should be similar. In fact, 75 μM iron and 300 μM copper are required to restore DNA replication rates to control levels (Fig. 1), while 200 μM iron and 200 μM copper are required to inhibit binding to p50 (Fig. 7). This observation may indicate that mimosine does, indeed, have an additional effect on DNA replication that does not operate through p50.

p50 is a soluble protein that is present in at least 10 000 copies per cell (calculated from the specific activity of the ^3H -mimosine preparation and the amount cross-linked to p50 at saturation; P. J. Mosca, unpublished observations). It is unlikely that p50 is a component of ribonucleotide reductase, whose subunit sizes are 86 and 43 kDa (16). p50 is present in both the nuclear and cytoplasmic fractions, and is therefore not likely to be a transport protein. Furthermore, ^3H -mimosine binding activity does not vary markedly as a function of cell cycle position, suggesting that p50 is probably not a cyclin. Finally, we have not been able to precipitate p50 with an antibody to the tumor suppressor, p53 (P. J. Mosca, unpublished observations). Thus, it is unlikely that p53 is the cellular target of mimosine.

Until we are able to purify p50 and determine its primary sequence, we can only guess what this mimosine-binding protein might be. However, several observations suggest that, rather than inhibiting an origin-binding protein as we had originally hoped, mimosine might interfere with some other downstream target that is required to establish replication forks.

For example, it has been shown that mimosine does not inhibit DNA replication in cleaving *Xenopus laevis* embryos, when the nucleotide pool levels are sufficiently high to sustain several rounds of DNA replication (M. L. DePamphilis, personal communication; R. M. Benbow, personal communication). Additionally, we have shown that mimosine has no effect on SV40 replication when added directly to an *in vitro* assay (R. M. Kalejta and J. L. Hamlin, submitted for publication). These observations could be used to argue that mimosine inhibits DNA replication simply by lowering nucleotide pools.

However, extracts prepared from mimosine-treated cells are compromised in their ability to support SV40 replication *in vitro* (R. M. Kalejta and J. L. Hamlin, submitted for publication). Furthermore, none of the inhibitors that function by lowering pools (e.g., hydroxyurea, 5'-fluorodeoxyuridine or methotrexate) are able to completely prevent replication fork progression *in vivo*. We and others have shown that all of these drugs are extremely leaky—whether added to cells as they approach the G_1/S boundary, or to log populations in which cells at all intervals of the S period would be present (12,14; J. L. Hamlin, unpublished observations). In contrast, mimosine is extraordinarily effective at preventing replication fork set-up at the beginning of the S period (11,12); yet, when delivered to cells in the middle of S, the drug appears to allow forks to progress to their eventual destinations, albeit more slowly than in the absence of mimosine (10,12).

Finally, when cells are allowed to traverse the G_1 period in the presence of mimosine and are arrested at the beginning of the S period for longer and longer time intervals, the initial rate of DNA synthesis extrapolates to zero regardless of the length of the block (11), arguing that replication forks have not been formed in the presence of mimosine. When this experiment is repeated with either hydroxyurea (a ribonucleotide reductase inhibitor; 16), aphidicolin (an inhibitor of several DNA polymerases; 15,35), or

cytosine arabinoside (a leaky chain terminator; 36), the initial rate of DNA synthesis increases in direct proportion to the length of the block (11, and L. Mesner and J. L. Hamlin, unpublished observations). Therefore, among these agents, mimosine is the only one that appears to prevent the formation of replication forks.

Until the target protein is purified and identified, we will not understand how the myriad observations on this unique and extremely efficacious replication inhibitor fit together. However, the process of sorting out the pieces of the puzzle may lead to important new uses for this compound (some of which have already been suggested; 18,31,37,38). In particular, the current cancer chemotherapeutic regimens suffer from a number of problems, including the lack of truly effective replication inhibitors and the development of resistance to the drugs that are effective. Mimosine could be a valuable new addition to the list of chemotherapeutic agents once its mechanism of action is understood, since it is an extremely efficacious inhibitor and since we were able to achieve only a 10-fold increase in drug-resistance after a selection regimen lasting more than one year.

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