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Mimosine Arrests the Cell Cycle after Cells Enter S-Phase

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L-Mimosine (*β-N*-[3-hydroxy-4-pyridone]-*α*-aminopropionic acid)—a rare amino acid derived from *Mimosa* and *Leucaena* plants—arrests cells reversibly late during G1 phase or at the beginning of S-phase. If mimosine were to arrest cells immediately before S-phase, it would provide a superb tool for the investigation of the initiation of DNA synthesis. Therefore, we reexamined the point of action of mimosine. Mitotic HeLa cells were released into 200 *μM* mimosine and grown for ~10 h to block them, before the cells were permeabilized and the amino acid removed by washing them thoroughly. On addition of the appropriate triphosphates, DNA synthesis—measured by the incorporation of [^{32}P]dTTP—began immediately; as it is known that such permeabilized cells cannot initiate DNA synthesis but can only resume elongating previously initiated chains, mimosine must arrest after DNA synthesis has begun. Moreover, cells grown in mimosine assembled functional replication factories—detected by immunolabeling after incorporation of biotin–dUTP—that were typical of those found early during S-phase. Disappointingly, it seems that mimosine—like aphidocolin—blocks only after cells enter S-phase.

INTRODUCTION

L-Mimosine (*β-N*-[3-hydroxy-4-pyridone]-*α*-aminopropionic acid) is a rare amino acid derived from *Mimosa* and *Leucaena* plants [1]. Farm animals fed on *Mimosa* become ill and the toxic agent—later identified as mimosine—has been shown to have a wide range of effects; it inhibits various mammalian enzymes *in vitro* (including alkaline phosphatase, tyrosinase, dopamine *β*-hydroxylase, deoxyhypusyl hydroxylase, and histone H1 kinase; reviewed in Ref. 2), it chelates transition metal ions [3] and binds—in the absence of excess iron or copper ions—to a 50-kDa protein [4, 5], while high concentrations disrupt chromatin [6–8].

Recently mimosine has been shown to arrest cells (reversibly) late in the G1 phase of the cell cycle [9] and so it has found increasing use as a synchronizing agent (e.g., Refs. 10–13). However, it has been difficult to relate the inhibitory effects described above to the cell cycle [5] and there has also been controversy as to precisely where the amino acid blocks. Some authors have concluded that it blocks progression somewhere between the point that cells become committed to enter S-phase and the beginning of DNA synthesis (e.g., Refs. 9, 10, 14–18) while others suggest that it acts only after synthesis has begun [5, 19, 20].

If mimosine were to arrest cells immediately before S-phase, it would provide a superb tool for the investigation of the initiation of DNA synthesis, which is hampered by the lack of any technique that provides good synchrony immediately before the G1/S phase border. Therefore, we have reexamined the point of action of mimosine. As much of the controversy could have arisen from the use of high concentrations of the amino acid over long periods, we deliberately used low concentrations for short periods. This required the use of cells presynchronized at mitosis. Moreover, the time taken for passage of mimosine into, and out of, cells then becomes relatively long compared with the period of exposure, so we permeabilized the cells to facilitate exchange. Disappointingly, we find that mimosine arrests progression through the cycle only after DNA synthesis has begun, much like aphidicolin [21].

METHODS

Cell culture, synchronization, encapsulation and permeabilization. Suspension cultures of HeLa cells were grown in minimal essential medium supplemented with 9% fetal calf serum and synchronized in mitosis using thymidine and nitrous oxide [22, 23]: cells were blocked in S-phase (2.5 m*M* thymidine, 22 h), washed carefully in PBS, regrown for 4 h in fresh medium, arrested in mitosis using nitrous oxide at high pressure (8 h, >98% in mitosis), and regrown for the periods indicated. Cells were encapsulated in microbeads of agarose and permeabilized using streptolysin O in a "physiological" buffer (PB) as described by Hozák *et al.* [24]. Intact cells were washed by three cycles of pelleting and resuspension in 50 ml growth medium; permeabilized cells were washed by three cycles of pelleting and resuspension in >10 vol PB.

In vivo replication assays. Cells were grown (~0.7×10^6/ml) in [*methyl*-^{3}H]thymidine (500 nCi/ml; ~40 Ci/mmol; Amersham) before duplicate samples (5 ml) were collected, the cells pelleted, the pellet

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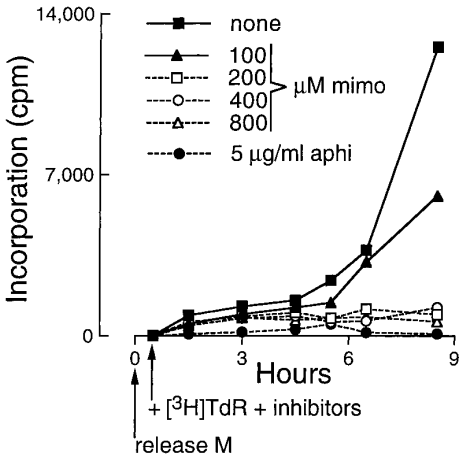

FIG. 1. Inhibition of DNA synthesis by mimosine is concentration dependent. HeLa cells were released from mitosis (M), regrown in [^{3}H]thymidine ± mimosine or aphidicolin, and the amount of ^{3}H incorporated into acid-insoluble material measured at various times.

dissolved in 100 μl 2% SDS and incubated (2 h; 37°C) before the whole sample was spotted on to GF/C discs (Whatman), and acid-insoluble radioactivity estimated by scintillation counting [25].

In vitro replication assays. Cells were encapsulated (~2 × 10^6 cells/ml beads), permeabilized with streptolysin O, and then allowed to incorporate [α-^{32}P]dTTP (~3000 Ci/mmol; Amersham) or biotin-16–dUTP essentially as described by Hozák *et al.* [26]. Radiolabeling reactions contained 1.1 m*M* ATP, 0.1 m*M* CTP, GTP, and UTP, plus 0.25 m*M* dATP, dCTP, and dGTP, plus 2.5 μM [^{32}P]dTTP (40 μCi/ml). For immunofluorescence, reactions contained 10 μM biotin-16–dUTP (Boehringer) instead of dTTP.

Immunofluorescence. Proliferating cell nuclear antigen (PCNA) was visualized as follows. Encapsulated cells (~10^6 cells/ml beads) were permeabilized (5 min, 4°C) using 0.5% Triton X-100 in PB, washed 3× in PB, fixed (15 min, 4°C) in methanol (precooled to −20°C), washed 4× in AB (4°C), incubated with mouse anti-PCNA (1/1000 dilution, 2 h, 4°C; Oncogene Science), rewashed 4× in PB, incubated with sheep anti-mouse antibody conjugated with FITC (1/1000 dilution, 12 h, 4°C; Amersham), rewashed 4× in AB, and mounted under coverslips in Vectashield (Vector Labs). AB is PBS with 0.5% BSA and 0.2% Tween 20. Photographs were taken on a Zeiss Axiophot using Kodak T-max 400 film.

After incorporating biotin–dUTP, beads were washed 4× in PB, incubated in Triton X-100 (0.5%, 5 min, 4°C), rewashed 3× in PB, fixed (15 min, 4°C) in 5% paraformaldehyde in PB, washed 4× in AB, incubated with a goat anti-biotin antibody (2 μg/ml in AB, 2 h, 4°C; Sigma), washed 4× in AB, incubated with donkey anti-goat antibody conjugated with FITC (1/1000 dilution, 12 h, 4°C; Jackson Immuno-Research), washed 4× in AB, and photographed as above.

RESULTS

Mimosine Arrests Cells at the G1/S Phase Border

More than 95% HeLa cells can be arrested in mitosis using nitrous oxide at high pressure [22]; after releasing the pressure, the now synchronized cells progress normally through the cell cycle. (See Refs. 23 and 26 for a detailed analysis of the resulting cell cycle.) If such mitotically arrested cells are regrown in [^{3}H]thymidine, the amount of ^{3}H incorporated into acid-insoluble material reflects the amount of DNA synthesis, which remains low for the first 4.5 h as cells progress through G1 phase (Fig. 1, closed squares). Synthesis by the minority of unsynchronized cells in the population accounts for most of this low background, which varies from experiment to experiment depending on the degree of synchrony. After 4.5 h, the first cells enter S phase and the incorporation increases. Increasing concentrations of mimosine progressively reduce this later incorporation until it disappears, but the highest concentration cannot completely abolish the earlier (background) incorporation; mimosine is more effective at inhibiting entry into S-phase than it is at inhibiting DNA synthesis [9, 17]. In all subsequent experiments, the lowest inhibitory concentration of mimosine (i.e., 200 μM) is used. The onset of S-phase was also inhibited when 200 μM mimosine was added up to 3.5 h after mitosis, but then later addition had progressively less effect (not shown). As expected, aphidicolin inhibited efficiently both background synthesis and entry into S-phase (Fig. 1, closed circles; see also Ref. 21).

The Reversibility of Mimosine Arrest

The inhibition of entry into S-phase by mimosine is reversible; cells arrested with mimosine continue to progress into S-phase when the amino acid is removed (Fig. 2).

We next tried to determine whether mimosine blocked progression before, or after, the point where aphidicolin acts. Mitotic cells were grown in mimosine for 10.5 h to arrest them, washed, and regrown in [^{3}H]-thymidine; they soon began to synthesize DNA, but

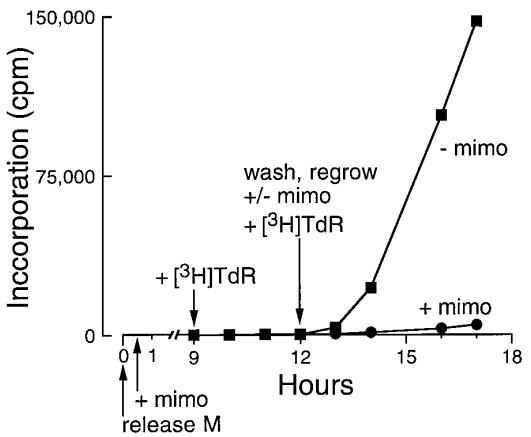

FIG. 2. Inhibition by mimosine is reversible. Cells were released from mitosis (M) into mimosine (200 μM) and [^{3}H]thymidine added after 9 h. Twelve hours after mitosis, cells were washed and returned to medium containing [^{3}H]thymidine ± mimosine. The amount of ^{3}H incorporated into acid-insoluble material was determined at the times indicated.

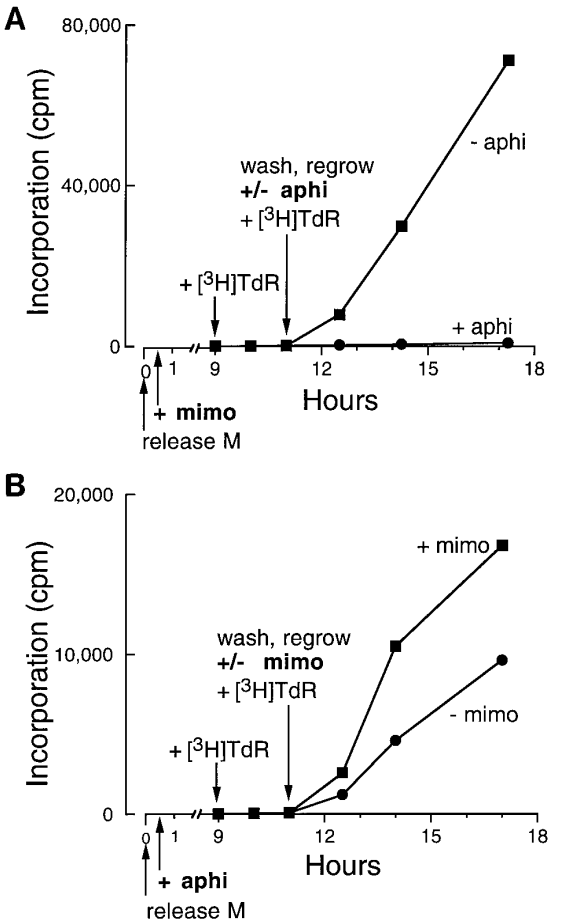

FIG. 3. Mimosine will not halt cells released from aphidicolin arrest, but aphidicolin halts cells released from mimosine arrest. Cells were released from mitosis (M) into mimosine (200 μM) or aphidicolin (5 μg/ml) and grown for 9 h and [^{3}H]thymidine added. Two hours later, cells were washed and regrown in [^{3}H]thymidine ± the other inhibitor, and the amount of ^{3}H incorporated into acid-insoluble material determined at the times indicated.

aphidicolin inhibited this synthesis (Fig. 3A). Strikingly different results were obtained when the sequence with which the drugs were administered was reversed. As expected, switching aphidicolin-arrested cells to drug-free medium allowed incorporation (Fig. 3B, −mimo), but mimosine increased this further (Fig. 3B, +mimo); not only does mimosine fail to maintain the inhibition, it also stimulates incorporation compared with the control grown in the absence of any drug (Fig. 3B). Such a stimulation—which has been seen under other conditions [27, 28]—is difficult to explain unless the amino acid affects precursor pools.

The failure of mimosine to maintain the inhibition of DNA synthesis has been used as evidence that it acts before aphidicolin [9, 14, 15]. However, this failure could result either if mimosine acts at the same point as aphidicolin, or if it acts later but enters cells slowly.

Mimosine Arrests Cells after DNA Synthesis Has Begun

Problems associated with slow entry (and exit) of mimosine into cells can be minimized by permeabilizing the cells with the bacterial toxin, streptolysin O [29, 30]. Such permeabilized cells are fragile, so they were also encapsulated in agarose microbeads (25–150 μm diameter) to protect them during subsequent manipulation. Small molecules—like mimosine and DNA precursors—can diffuse in and out of such encapsulated and permeabilized cells within seconds. For example, we have previously shown that—on addition of the appropriate triphosphates—permeabilized cells in S-phase immediately resume DNA synthesis at the rate found *in vivo;* preexisting nascent chains are elongated but no new synthesis is initiated [31].

If mimosine arrests before entry into S-phase, arrested and permeabilized cells should be unable to synthesize DNA; if it arrests after entry, synthesis should resume immediately. Therefore cells were released from mitosis into mimosine and grown for 10 h; then the arrested cells were encapsulated, permeabilized in a "physiological" buffer, and supplied with the appropriate triphosphates. [^{32}P]dTTP incorporation began immediately, whether or not mimosine was present (Fig. 4). Clearly, such mimosine-arrested cells have already initiated the synthesis of new chains and so have entered S-phase.

Mimosine Arrests Cells during, or after, the Assembly of Replication Factories

DNA synthesis does not occur diffusely throughout euchromatin in mammalian nuclei but is concentrated

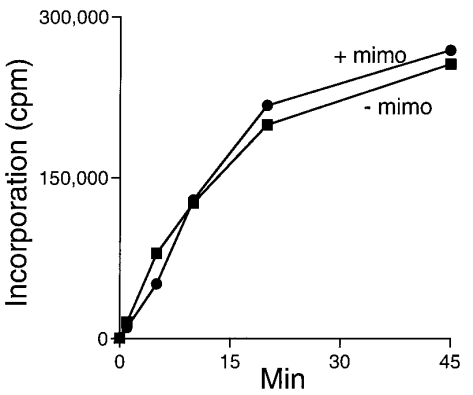

FIG. 4. Mimosine arrests cells after DNA synthesis begins. Cells were released from mitosis into mimosine (200 μM), grown for 10 h, encapsulated in agarose (in the continued presence of mimosine), permeabilized with streptolysin O, and allowed to synthesize DNA *in vitro* in the presence (circles) and absence (squares) of mimosine. [^{32}P]dTTP was included during synthesis, thereby allowing the incorporation of radio-label into acid-insoluble material to be measured.

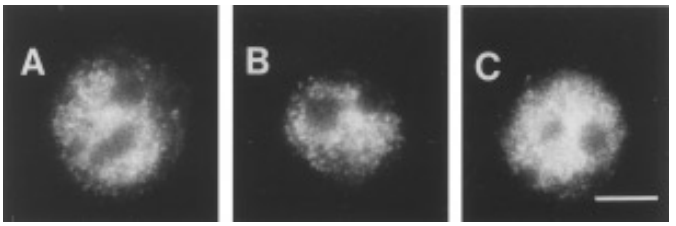

FIG. 5. Mimosine arrests cells during, or after, the incorporation of PCNA into replication factories. Cells were released from mitosis into (A) mimosine (200 μM) or (B) aphidicolin (5 μg/ml), grown for 9 h, encapsulated in agarose (in the continued presence of mimosine or aphidicolin) and lightly fixed with methanol. This removes a soluble pool of PCNA that is spread throughout nuclei but leaves PCNA within factories [34]. Then PCNA was indirectly immunolabeled. (C) Control cell (untreated with any drug) in early S-phase. Labeling patterns in A–C are similar. Bar, 5 μm.

in discrete replication "foci" or "factories" (e.g., Refs. 24, 32, 33). The replicative enzymes DNA polymerase α and PCNA are detected by immunofluorescence as a diffusely spread and inactive pool early during G1-phase, but as cells progress toward S-phase this pool is incorporated into the factories [26, 34]. Therefore we determined whether mimosine (and aphidicolin) arrested cells before, or after, the incorporation of PCNA into these factories.

Mimosine- and aphidicolin-arrested cells were encapsulated in agarose, fixed and indirectly immunolabelled using antibodies directed against PCNA. PCNA within both types of cell had the pattern typical of early S-phase cells (Fig. 5, compare A and B with C). Therefore, mimosine—like aphidicolin—arrests cells during, or after, the assembly of PCNA into the factories.

We next determined whether these factories were capable of replicating. Mimosine- and aphidicolin-arrested cells were encapsulated in agarose as before, permeabilized, and allowed to elongate nascent DNA in the presence of biotin–dTTP; then sites containing incorporated biotin were immunolabelled with fluorescently tagged antibodies. Figure 6 illustrates the resulting patterns, which are typical of cells in early S-phase [26, 33]. Again, mimosine—like aphidicolin—arrests cells as, or after, factories become active.

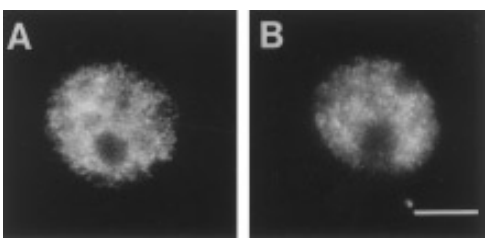

FIG. 6. Mimosine arrests cells during, or after, factories become active. Cells were released from mitosis into (A) mimosine (200 μM) or (B) aphidicolin (5 μg/ml), grown for 9 h, encapsulated in agarose, permeabilized, and allowed to make DNA in the presence of biotin–dTTP; then sites containing incorporated biotin were indirectly immunolabeled and photographed using a fluorescence microscope. Labeling patterns in A and B are similar. Bar, 5 μm.

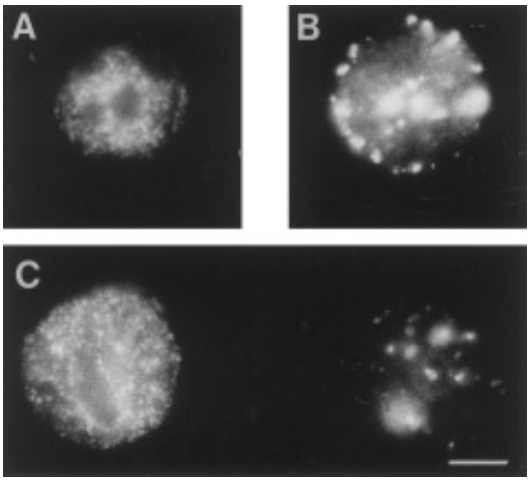

FIG. 7. Mimosine slows the evolution of factories. Cells were synchronized with aphidicolin at the beginning of S-phase, released from arrest, and regrown for 2 h; then mimosine was added and the cells grown for a further 6 or 10 h before sites of biotin incorporation were immunolabeled. Patterns typical of (A) ~90% cells after 6 h in mimosine, (B) untreated controls (in late S-phase) after 6 h, and (C) ~40% cells after 10 h in mimosine (mid- and late S-phase cells are shown on the left and right, respectively). Bar, 5 μm.

Mimosine Slows the Remodeling of Factories

As cells progress through S-phase, factories change in number, size, and distribution. Early during S-phase they are small, discrete, and distributed throughout extranucleolar regions, and then they enlarge and become concentrated around the nuclear periphery, before the giant factories of late S-phase form in the interior (e.g., Refs. 26, 33, 35, 36). Therefore we determined how mimosine affects the remodeling of factories.

Cells were synchronized with aphidicolin at the beginning of S-phase, released from the arrest, and grown for 2 h, before mimosine was added and the cells regrown for a further 6 h before sites of biotin incorporation were immunolabeled. Then ~90% had the pattern typical of early S-phase cells immediately after release from the aphidicolin block (Fig. 7A; compare with Fig. 6B), which was quite unlike the late S-phase pattern seen in untreated controls (Fig. 7B). If the cells were grown for 10 h (instead of 6 h) in mimosine, then ~40% had patterns typical of mid S-phase and another ~40% had late S-phase patterns (Fig. 7C, left and right, respectively); this is to be compared to controls grown in the absence of mimosine where most cells had left S-phase so that only ~1% were labeled (not shown). Clearly, mimosine slows the remodeling of factories.

DISCUSSION

Mimosine Arrests after DNA Synthesis Has Begun

Mimosine has often been used to block progression of cells around the cell cycle; however, the precise point at which this rare amino acid acts has been controversial (see Introduction). Several interrelated factors have contributed to this controversy. The amino acid is not very potent, requiring the use of high concentrations (i.e., 100–1200 μM) for extended periods (i.e., 14–48 h). As the block is imperfect, cells continue to progress—albeit slowly—around the cycle during the block. And then when the block is reversed by washing out the mimosine, residual levels may continue to inhibit progression. Therefore we presynchronized our cells at mitosis using an efficient procedure (i.e., using successive thymidine and nitrous oxide blocks) so that a short exposure of only 9–10 h to 200 μM mimosine effectively blocked most cells in the population near the G1/S phase border. We also reversed the block efficiently by permeabilizing the cells and then thoroughly washing out the mimosine.

If mimosine arrests cells immediately before S-phase as has often been claimed, it would provide a superb tool for investigating the initiation of DNA synthesis. Disappointingly, our results—like those of Dai *et al.* [19], Mosca *et al.* [5], and Gilbert *et al.* [20]—show that mimosine acts like aphidicolin to block cells after DNA synthesis has initiated (i.e., after primer synthesis and probably at the stage when nascent DNA chains are being elongated). [See Ref. 28 for a discussion of how various inhibitors—including mimosine, aphidicolin, hydroxyurea, cytosine arabinoside, and ciclopyrox olamine—might block the cycle.] Two different kinds of experiment support this conclusion. In the first, mitotic cells were released into mimosine (or aphidicolin) to block them before the drugs were removed by permeabilization and washing; on addition of the appropriate triphosphates, DNA synthesis—measured by the incorporation of [^{32}P]dTTP—began immediately (Fig. 4). As we have previously shown that such permeabilized cells cannot initiate DNA synthesis but can only resume elongating previously initiated chains [23, 31], this means that mimosine (and aphidicolin) arrest the cycle after DNA synthesis has begun. A second—similar—experiment involved monitoring the construction of functioning replication "factories" in which most S-phase DNA synthesis occurs [24, 26, 32]. Cells grown in mimosine (and aphidicolin) assembled such factories—detected here by immunolabelling after incorporation of biotin–dUTP—that were able to replicate DNA and which were typical of those found early during S-phase (Fig. 6).

This disappointing conclusion has one important *caveat*. It is, of course, possible that mimosine blocks *before* DNA synthesis begins; then—because it is a "leaky" inhibitor—the (partially blocked) cells may progress into S-phase even in the presence of the amino acid to give the results that we see. Unfortunately it is currently impossible to eliminate this possibility completely but the use of 800 μM mimosine (as in Fig. 4) did not prevent both the immediate resumption of DNA synthesis by the permeabilized cells or the assembly of functional factories (not shown). Note that if mimosine blocks during G1 phase, it must also have a second point of action during S-phase (see below).

The Mode of Action of Mimosine

All our results are consistent with the simple view that mimosine slows elongation of nascent DNA chains and that it does so less efficiently than aphidicolin (see also Ref. 17). For example, it reduces—but does not eliminate—S-phase synthesis (Fig. 1, "background" synthesis) and it slows the normal development of small factories into large factories during S-phase (Fig. 7).

Precisely how mimosine might slow elongation is obscure, but it has recently been suggested that it might act by chelating iron ions, thereby inhibiting the enzyme ribonucleotide reductase [5, 19]. This enzyme converts NTPs to dNTPs and is largely responsible for determining the intracellular concentrations of dNTPs [37]. Two of our results are consistent with this hypothesis: (i) mimosine inhibits by some mechanism that is readily reversed by permeabilization (Figs. 4 and 6) and (ii) it will not block cells released from an aphidicolin arrest, whereas aphidicolin will block cells released from mimosine-arrest (Fig. 3; see also Refs. 9, 14, 15). However, a precise molecular description of how this rare amino acid inhibits must await further experimentation.

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