Azidocytidine Is a Specific Inhibitor of Deoxyribonucleotide Synthesis in 3T6 Cells*

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Azidocytidine is a nucleoside analogue containing an N₃ group in place of the OH group at position 2' of cytidine (1). The 5'-diphosphate of azidocytidine (azido-CDP) is a suicide inhibitor of the enzyme ribonucleotide reductase and inactivates the B2 subunit of the enzyme from Escherichia coli by scavenging its tyrosyl radical (2, 3). The elucidation of the mechanism of this process was of considerable importance for an understanding of the radical mechanism of ribonucleotide reductase (3-6).

Addition of azidocytidine to rapidly growing cells in tissue culture resulted in an inhibition of DNA synthesis and cell death (7). Rather unexpectedly, a closer analysis revealed that ribonucleotide reductase apparently was not the primary target of inhibition which, instead, seemed to be caused by a block in the initiation of DNA replication (8). Subsequent experiments modified this view in that we found that azidocytidine, after phosphorylation in 3T6 cells, had a dual effect: low doses of the analogue primarily inhibited DNA strand elongation (but not initiation of strands), while at higher doses also the reductase was affected (9). A mutant cell line, lacking the enzyme deoxycytidine kinase, was completely resistant to the analogue, demonstrating that phosphorylation was required both for the inhibition of strand elongation and of the reductase. A highly puzzling aspect was, however, that azido-CTP (the presumptive nucleotide analogue acting as inhibitor of chain elongation) had no effect on DNA replication in in vitro systems from 3T6 cells (10). The mechanism by which azidocytidine inhibited strand elongation therefore remained unresolved and paradoxical.

The preparations of azidocytidine used in earlier work were obtained by chemical synthesis and were better than 99% pure as judged from HPLC analysis. However, we recently found that arabinosyl cytosine, at a level of 0.1-0.3%, was a consistent contaminant. Since arabinosyl cytosine is a highly potent inhibitor of DNA replication (11, 12), it seemed possible that it was responsible for the inhibition of chain elongation. We now demonstrate that after further purification by HPLC, azidocytidine, free from arabinosyl cytosine, no longer inhibits DNA strand elongation in 3T6 cells. The effects of the pure drug now mimic those of hydroxyurea, a known specific inhibitor of ribonucleotide reductase, and thus azidocytidine appears to inhibit DNA synthesis exclusively via the known effect of azido-CDP on ribonucleotide reductase. Azidocytidine could therefore be used as a specific inhibitor of ribonucleotide reductase in studies concerning the size and turnover of deoxynucleoside triphosphates (dNTP) pools in intact cells, and these experiments complement and amplify similar studies with hydroxyurea (13).

EXPERIMENTAL PROCEDURES

Materials—Commercially available radioactive nucleosides and nucleotides were obtained from the Radiochemical Centre, Amersham, England. 3H labeling of azidocytidine was done by the tritium labeling service at Amersham Corp. The material was purified by chromatography on Dowex 1 and HPLC. To this purpose, 100 mCi of the labeled material dissolved in 25 ml of water was adsorbed to a column of Dowex 1 (12 ml, OH form). The column was washed extensively with water (40 ml) until no more radioactivity appeared in the effluent. Azidocytidine (15 μmol) corresponding to 10% of the applied radioactivity was then eluted with 30% methanol, with a specific activity of 0.56 × 10⁶ cpm/nmol. A short time before use, the nucleoside was further purified by HPLC chromatography as described below.

Nonlabeled nucleosides were from Serva; deoxynucleoside triphosphates from F-L Biochemicals; hydroxyurea from Sigma; and azidocytidine, poly[d(A-T)], poly[d(IC)], and DNA polymerase I from E. coli from Boehringer Mannheim.

Growth and Incubation of Cells—3T6 mouse fibroblasts used in our experiments were obtained from frozen ampuls and grown in Dulbecco's modified Eagle's medium containing 10% horse serum as described (13). The cells were occasionally screened for contamination by mycoplasma and found to be negative.

Radioactive labeling of cells with 0.5 μM [3H]thymidine or [5-3H]cytidine was done with 2-day-old cultures containing between 0.5 and 1.0 × 10⁶ cells on 5-cm Petri dishes (14), as indicated in the appropriate text to figures.

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Azidocytidine Inhibits Ribonucleotide Reductase

Growth inhibition studies were started by seeding 0.5 x 10^6 cells in complete medium onto 6-cm dishes in the presence of various concentrations of azidocytidine. After 72 h of incubation at 37°C, the medium was removed, and the cells were washed with three changes of Tris/saline and dissolved in 1 ml of 1 M NaOH. The amount of growth was calculated from a standard curve (15). Analyses of Deoxyribonucleotides and DNA—Nucleotide pools were separated from DNA by extraction of cells with either 60% methanol or 0.5 M HClO4. The latter procedure was used for the decay experiment depicted in the legend to Fig. 6. The methanol extracts were used for the determination of the size of dNTP pools and their specific activities (16-19). The extracted cell pellet was used for the determination of incorporation of radioactivity into DNA and RNA (9).

Phosphorylation of Azidocytidine or Arabinosyl Cytosine—Growing 3T6 cells (1 x 10^6/cm²) were incubated with either radioactive cytidine analogue in the presence of various amounts of the other nonlabeled analogue or deoxycytidine. At the indicated time, the cell layers were washed three times with ice-cold Tris/saline, and the cells from each dish were extracted with 2 ml of 60% methanol (16). After standing over night at -20°C, the extracts were centrifuged and the supernatant solutions were evaporated to dryness in a vacuum. The dry residues were dissolved in 0.2 ml of 20 mM Tris-HCl, pH 7.5, and 10-µ1 portions were chromatographed on polyethylenimine-cellulose TLC plates with 0.2 M LiClO4 to separate nucleosides from nucleotides (20). The areas of the chromatograms containing the nucleotide fractions were cut out and placed into scintillation vials. The nucleotides were extracted for 2 h at room temperature with 1 ml of 1 M HCl. Then 10 ml of Insta-Gel was added, and the radioactivity in the vials was determined directly in a Packard scintillation counter.

Incorporation of [5-3H]Cytidine into Cytidine and Deoxycytidine Phosphates—The total radioactivity incorporated from cytidine into the sum of deoxycytidine phosphates, a rough measure of the in situ activity of ribonucleotide reductase, was determined as described (9). Briefly, methanol extracts from cells were hydrolyzed with 1 M HClO4 and, after neutralization, dCMP was separated from CMP on Dowex 50 columns (21). The total radioactivity present in the dCMP fraction was then a measure of the in situ activity of the reductase. The total radioactivity of the CMP fraction reflected the phosphorylation of cytidine and in the appropriate instances the possible effect of an inhibitor of this parameter.

Release of [3H]Cytidine from [5-3H]Cytidine—The recovery of tritium in water during incubation of cells with a nucleoside labeled at position 5 of the pyrimidine ring is a measure of the in situ activity of thymidylate synthetase (22, 23). Measurements were done as described earlier (13).

Analysis and Purification of Azidocytidine by HPLC—The analysis of azidocytidine described in the legend to Fig. 1 was carried out by chromatography on an analytical column reversed-phase column by isocratic elution with 50 mM triethylammonium acetate, pH 5.5, containing 5% methanol (24, 25). For the purification of both unlabeled and labeled azidocytidine, a semipreparative C18 column (10 x 250 mm) was eluted with 20% methanol at a rate of 3 ml/min. Simultaneous recording of the absorption at 254 and 280 nm facilitated the identification of the contaminating arabinosyl cytosine since the 280/254 ratio for this nucleoside at neutral pH was 1.1 as compared to a value of 0.9 for azidocytidine, cytidine, or deoxycytidine.

RESULTS

We began to suspect that a contaminant of azidocytidine might have complicated our earlier experiments when it was found that two separate preparations of the drug showed considerable variations in their growth inhibitory effects. Analysis by HPLC demonstrated that both preparations were better than 99% pure but that the more potent drug contained about three times the amount of an impurity moving as arabinosyl cytosine during HPLC (cf. insets to Fig. 1, A and B). The two preparations inhibited cell growth to 50% at concentrations of 4 and 10 µM, respectively (Fig. 1, A and B). Azidocytidine and the more potent drug inhibited cell growth to 50% at concentrations of 20 µM (Fig. 1C). Clearly, arabinosyl cytosine might have been responsible for some of our earlier results, and it was decided to reinvestigate the effects of azidocytidine on dNTP and DNA synthesis with the preparation of the drug purified by HPLC.

In a first experiment (Fig. 2), rapidly growing 3T6 cells were preincubated for 3 h with different concentrations of azidocytidine and then labeled with 0.5 µM [5-3H]cytidine or [methyl-3H]thymidine in the continued presence of the drug. At the end of the labeling periods, we determined the size of the dTTP and dCTP pools, their specific activities, and the rate of RNA (isotope incorporation from cytidine) and DNA (incorporation from thymidine or cytidine) synthesis. In the cytidine experiment, we also determined the total radioactivity present in all deoxycytidine phosphates, a measure of the in situ activity of ribonucleotide reductase, as well as the radioactivity present as cytidine phosphates.

The pool measurements depicted in Fig. 2A demonstrate that the dTTP pool increased in size almost 2-fold at the highest concentration of the drug, while dCTP decreased to about 50%. Incorporation of thymidine into dTTP was affected marginally by azidocytidine, while the specific activity of dCTP labeled from cytidine decreased continuously with increasing drug concentration and dropped to less than one-third. Fig. 2B demonstrates that azidocytidine did not affect incorporation of cytidine into cytidine phosphates or RNA synthesis, while DNA synthesis was inhibited profoundly. The decrease in DNA synthesis furthermore showed a striking parallelism with the decrease of the incorporation of radioactivity from cytidine into deoxycytidine phosphates. The latter curve reflects the inhibition of the in situ activity of ribonucleotide reductase by azidocytidine.

The data depicted in Fig. 2 differ strikingly from results of an identical experiment obtained earlier with a preparation of azidocytidine containing arabinosyl cytosine (9). While the earlier results suggested inhibition of DNA replication independent of ribonucleotide reductase, our present data can be fully explained by an inhibition of the reductase. The earlier results then depended on the contaminating arabinosyl cytosine. One puzzling aspect remained. Both azidocytidine and arabinosyl cytosine are apparently phosphorylated by the same enzyme, deoxycytidine kinase, and a large excess of azidocytidine might have been expected to prevent the phosphorylation of arabinosyl cytosine. To clarify this aspect, we investigated in the experiment depicted in Fig. 3 the effects of competition by nonlabeled nucleosides on the uptake and phosphorylation of labeled azidocytidine (A and C) or arabinosyl cytosine (B and D).

The phosphorylation of both labeled nucleosides was strongly depressed by deoxycytidine. Similarly, arabinosyl cytosine competed successfully for phosphorylation of azidocytidine. Since the competition could occur at several steps, starting at the transport of nucleosides into the cell (26) and involving the activities of several kinases, a closer kinetic analysis does not appear to be meaningful. However, as an example, in the experiment shown in Fig. 3C, 30 µM arabinosyl cytosine inhibited by approximately 50% the phosphorylation of 100-300 µM azidocytidine.

In contrast, azidocytidine had essentially no effect on the phosphorylation of arabinosyl cytosine, not even when present in 200-fold excess (Fig. 3D). The inability of azidocytidine to prevent the phosphorylation of arabinosyl cytosine fully explains why a small contamination by arabinosyl cytosine could have such a profound effect on DNA replication.

We next compared the effects of azidocytidine, hydroxyurea, and arabinosyl cytosine on dNTP and DNA synthesis. Hydroxyurea is well established as an inhibitor of ribonucleotide reductase (27-29), while arabinosyl cytosine inhibits...
Azidocytidine Inhibits Ribonucleotide Reductase

**FIG. 1.** Inhibition of growth of 3T6 cells by three preparations of azidocytidine. A and B show the effect of various concentrations of two separate commercial preparations of azidocytidine on the growth of 3T6 cells, measured as described under "Experimental Procedures." C shows an identical experiment with a preparation of azidocytidine further purified by HPLC. The insets give the analyses of the three preparations of HPLC. The positions of peaks 1–4 correspond to those of cytosine, cytidine, arabinosyl cytosine, and azidocytidine, respectively.

**FIG. 2.** Effects of azidocytidine on deoxynucleotide and DNA synthesis in 3T6 cells. Growing 3T6 cells (0.5 × 10⁶ cells/5-cm dish) were incubated at 37 °C for 3 h with different concentrations of azidocytidine. Parallel sets of cultures were then further incubated in the presence of the drug either with 0.5 μM [5-¹³C]cytidine for 60 min or with 0.5 μM [¹³C]thymidine for 30 min and used for the determination of pool sizes, specific activities of pools, and incorporation of isotope into cytidine and deoxycytidine phosphates as well as RNA and DNA. A shows the effects of the drug on pool sizes of dCTP (□—□) and dTTP (○—○) as well as the specific activities of dCTP (●—●, labeled from cytidine) and dTTP (△—△, labeled from thymidine). B shows the incorporation of cytidine into RNA (∇), cytidine phosphates (□), deoxycytidine phosphates (○), and DNA (■) as well as the incorporation of thymidine into DNA (●). The incorporation of cytidine into DNA was corrected for the observed decrease in the specific activity of the dCTP pool depicted in A.

**FIG. 3.** Competition between azidocytidine and arabinosyl cytosine for phosphorylation by 3T6 cells. Growing 3T6 cells (1.0 × 10⁶ cells/5-cm dish) were incubated with either labeled azidocytidine (55–200 cpm/pmol, A and C) or labeled arabinosyl cytosine (230 cpm/pmol, B and D). A shows the time course for the phosphorylation of 50 μM azidocytidine and the inhibitory effects of 80 μM arabinosyl cytosine or 13 μM deoxycytidine; B shows the phosphorylation of 4 μM arabinosyl cytosine and the effects of 165 μM azidocytidine or 13 μM deoxycytidine; C shows the inhibitory effects of two concentrations of arabinosyl cytosine (50 or 105 μM) on the phosphorylation of various concentrations of azidocytidine during a 110-min incubation at 37 °C; and D shows the effects of 500 μM azidocytidine or 3 μM deoxycytidine on the phosphorylation of arabinosyl cytosine during a 90-min incubation at 37 °C.

**FIG. 4.** DNA strand elongation (11, 12) without interfering with the reductase. Fig. 4 depicts, side by side, the effects of increasing concentrations of azidocytidine and hydroxyurea, respectively, on the size of the four dNTP pools as well as on DNA synthesis (incorporation of thymidine) and the recovery of ³H released as ³H₂O from [5-³H]cytidine. ³H₂O is released during the methylation of dUMP derived by deamination of dCMP obtained by reduction of [5-³H]CDP. This parameter therefore measures the combined in situ activities of ribonucleotide reductase, dCMP deaminase, and thymidylate synthetase.
The half-lives of the dTTP or dCTP pools, while inhibition of strand elongation by aphidicolin only marginally affected over. In the experiment depicted in Fig. 6, we now determined effect and depends on the decreased activities of ribonucleotide reductase and thymidylate synthetase caused by the urea but differed from arabinosyl cytosine. While all three labeling of deoxycytidine phosphates as a measure of the case, we also determined the effects of the analogue on experiments with arabinosyl cytosine are depicted in Fig. 5. In contrast, the dATP pool expanded on addition of arabinosyl cytosine but decreased with both hydroxyurea or azidocytidine.

When it comes to the release of $^3$H$_2$O, we can again note that the effect of azidocytidine was similar to that of hydroxyurea but differed from arabinosyl cytosine. While all three drugs decreased the release of $^3$H$_2$O, inhibition by hydroxyurea and azidocytidine was much more severe than by arabinosyl cytosine. Inhibition by arabinosyl cytosine is a secondary effect and depends on the decreased activities of ribonucleotide reductase and thymidylate synthetase caused by the inhibition of DNA strand elongation. It is similar in nature to the earlier demonstrated effect of aphidicolin (13). Hydroxyurea and azidocytidine, on the other hand, directly inhibit ribonucleotide reductase, turn off the flow of isotope from cytidine to dUMP, and thereby stop the release of $^3$H$_2$O completely. This interpretation is substantiated by comparing the effects on $^3$H$_2$O release and DNA synthesis: hydroxyurea and azidocytidine inhibit both processes to the same extent; with arabinosyl cytosine, the relative inhibition of DNA synthesis is much more severe.

An additional distinction between effects on ribonucleotide reductase and DNA strand elongation relies on the perturbation of the turnover of dNTP pools by an inhibitor (31). In other work from this laboratory (13), we found that a block of strand elongation by aphidicolin only marginally affected the half-lives of the dTTP or dCTP pools, while inhibition of the reductase by hydroxyurea essentially stopped their turnover. In the experiment depicted in Fig. 6, we now determined the effects of azidocytidine, arabinosyl cytosine, and hydroxyurea on dTTP turnover. Three parallel sets of cultures were prelabeled with $[^3$H]thymidine for 30 min in the presence of either inhibitor to label the dTTP pool. The isotopic medium was replaced with conditioned medium containing nonlabeled thymidine and, at different time intervals thereafter, the radioactivity of the dTTP pool was determined. The decay of isotope then measured the turnover of the pool (14). Fig. 6 shows that the decay in the presence of azidocytidine closely follows that with hydroxyurea, but differs widely from that with arabinosyl cytosine, again supporting the conclusion that azidocytidine primarily blocks ribonucleotide reductase and that the inhibition of DNA replication is a secondary effect.

**DISCUSSION**

Earlier experiments using azidocytidine as an inhibitor of DNA synthesis in intact cells gave conflicting results (7–10). It now appears that the confusion was caused by a trace contamination of the commercial preparation of azidocytidine with arabinosyl cytosine. The results obtained with azidocytidine further purified by HPLC can now be fully explained by the known inhibition of ribonucleotide reductase by the diphosphate of the nucleoside. A decisive finding was the effect of the nucleoside on the flow of isotope from labeled cytidine via dNTP pools into DNA. Earlier experiments (9, 32) established the usefulness of this approach to distinguish between inhibition of dNTP synthesis and dNTP utilization. The present data, shown in Fig. 2, demonstrate a striking parallelism between inhibition of CDP reduction and DNA replication and are in sharp contrast with our earlier data obtained in a similar experiment with a contaminated preparation of azidocytidine (9).

Additional evidence for the primary inhibition of the reductase is provided by the experiments depicted in Figs. 4–6. Azidocytidine affects dNTP pool sizes and dTTP pool turnover in a manner similar to hydroxyurea, a known inhibitor of the reductase, but dissimilar to arabinosyl cytosine or aphidicolin (13), inhibitors of DNA strand elongation. This also applies to the release of tritium from $[^5$H]cytidine which
Azidocytidine Inhibits Ribonucleotide Reductase

**Fig. 5. Effect of arabinosyl cytosine on the size of dNTP pools, synthesis of DNA, and in situ activities of thymidylate synthetase and ribonucleotide reductase.** The experiment was similar to the one described in the legend to Fig. 4 except that the incorporation of [5-3H]cytidine into DNA was used to measure DNA synthesis and that the incorporation of [5-3H]cytidine into deoxycytidine phosphates was monitored to measure the effect of arabinosyl cytosine on the in situ activity of ribonucleotide reductase. A shows the effect on pool sizes, and B on DNA synthesis (V) and in situ activities of the reductase, as measured by isotope incorporation into deoxycytidine phosphates (W) and thymidylate synthetase, as measured by release of 3H2O (O).

is a measure of the combined in situ activities of several enzymes involved in dTMP production, including ribonucleotide reductase. The combined evidence clearly indicates that ribonucleotide reductase is the primary target for the inhibition of DNA synthesis by azidocytidine.

Azidocytidine must be phosphorylated by a deoxycytidine kinase in order to exert its effects since cells lacking this enzyme activity are completely resistant to inhibition (9). The affinity of the kinase for the nucleoside appears to be low since relatively high concentrations are required to build up sizable amounts of the intracellular nucleotide pool (9). In the experiment described in Fig. 3, the synthesis of 100 pmol of azidonucleotides required a 100 \( \mu M \) concentration of the nucleoside in the medium, while 2 \( \mu M \) sufficed for the corresponding synthesis from arabinosyl cytosine. The intracellular radioactivity was in both instances a mixture of nucleotides, and the data thus reflect the combined action of several kinases. However, we believe that the activity of deoxycytidine kinase(s) was the limiting factor. There is evidence for the existence of more than one such kinase in mammalian cells (33), and this might explain some of the peculiarities concerning the interaction between azidocytidine and arabinosyl cytosine. While arabinosyl cytosine competed successfully for the phosphorylation of azidocytidine, even a 200-fold excess of the latter nucleoside had essentially no effect on arabinosyl cytosine phosphorylation. Such a result might be explained if the two nucleosides are phosphorylated by separate kinases and if the kinase that phosphorylates azidocytidine also can bind arabinosyl cytosine. However, more enzyme work is required to clarify this point. Under all circumstances, these results explain why the small amounts of arabinosyl cytosine contaminating the preparation of azidocytidine could be phosphorylated in the presence of a large excess of azidocytidine.

The efficient phosphorylation of arabinosyl cytosine only in part explains why this nucleoside on a molar basis inhibits DNA synthesis 1000-fold more effectively than azidocytidine (cf. data in Figs. 4 and 5). The triphosphate of arabinosyl cytosine (ara-CTP) competes with dCTP during DNA replication, while azido-CDP competes with ribo-CDP. The \( K_i \) value for azido-CDP was reported to be close to the \( K_i \) value for CDP (34), while ara-CTP appears to have a higher affinity for \( \alpha \)-polymerase than dCTP (35). In addition, the intracellular pools of cytidine phosphates are 10-fold larger than the pools of deoxycytidine phosphates (24). These circumstances all contribute to the efficient inhibition by arabinosyl cytosine.

Azidocytidine can now be used as a tool to inhibit specifically the activity of ribonucleotide reductase in intact cells. The data in Figs. 4–6 describing and comparing the effects of azidocytidine and arabinosyl cytosine on the in situ activity of dTMP synthetase and dTTP pool turnover relate to and complement similar more extensive studies from this laboratory (13) with hydroxyurea and aphidicolin. There we found that the complete inhibition of DNA strand elongation by aphidicolin decreased but did not stop de novo synthesis of pyrimidine dNTPs which under those circumstances were degraded and excreted as deoxyribonucleosides into the medium. The present data with arabinosyl cytosine, a second inhibitor of DNA strand elongation, are in line with these results. In

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**Fig. 6. Effects of azidocytidine, hydroxyurea, and arabinosyl cytosine on the turnover of the dTTP pool.** Parallel sets of growing 3T6 cells (0.2 × 10^6 cells/3-cm dish) were first incubated at 37 °C (control, ◇) with 0.7 mM azidocytidine (○), 1.5 mM hydroxyurea (□), or 7 \( \mu M \) arabinosyl cytosine (△) for 30 min. The cells were then labeled with 0.5 \( \mu M \) [3H]thymidine for an additional 30 min in the presence of the inhibitor, after which time the radioactive medium was replaced with conditioned medium, containing 0.5 \( \mu M \) cold thymidine (14) and inhibitor. At the time intervals indicated, samples were removed and the amount of remaining HClO4-extractable radioactivity was determined. At least 80% of this radioactivity was dTTP. The concentrations of inhibitors chosen in this experiment inhibited DNA synthesis by more than 95%.
Azidocytidine Inhibits Ribonucleotide Reductase

Contrast, during inhibition with hydroxyurea, the turnover of dNTPs ceased and deoxynucleosides were transported from the medium into the cells. Again, the present results with azidocytidine, a second inhibitor of the reductase, gave similar results. As a model to explain these results, we invoked the existence of substrate cycles between deoxynucleosides and their 5'-phosphate involving the converted action of kinases and phosphatases and regulating the transport of deoxynucleosides in and out of cells (30). Through their interplay with de novo synthesis, such cycles would participate in the regulation of the size of dNTP pools. The present work is in accordance with such a model.

Why does inhibition of ribonucleotide reductase stop DNA synthesis? Lack of one or more building blocks for DNA synthesis with necessity puts an end to replication, and earlier experiments with secondary mouse embryo cells (31) suggested such an explanation. In these cells, the dGTP pool was the smallest dNTP pool (3 pmol/10^6 cells) and all but disappeared within a few minutes after addition of hydroxyurea. In 3T6 cells, however, the dGTP pool was larger and in size equal to the dATP pool (20–25 pmol/10^6 cells each). Inhibition of the reductase primarily decreased the dCTP and dATP pools, but did not deplete them completely. The data of Fig. 4 might be taken to suggest that inhibition of DNA synthesis was more severe than expected from the decrease in the two dNTPs. Possible explanations involve a requirement of DNA synthesis for a critical level of one or several dNTPs and/or compartmentation of pools, such as was found for dCTP (14). Further work is in progress to find answers to these questions.

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