Evaluation of Ribonucleoside and Deoxyribonucleoside Triphosphate Pools in Cultured Leukemia Cells during Exposure to Methotrexate or Methotrexate Plus Thymidine

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ABSTRACT

Continuous exposure to inhibitory concentrations of methotrexate produces distinct rates of steady-state growth of murine leukemia L1210 and human leukemia CCRF-CEM cells in culture. Addition of thymidine to the medium produces reversal (6 to 40%) of this steady-state growth rate inhibition. This study utilized combinations of methotrexate and thymidine for an evaluation of the accompanying relationship between steady-state growth rate and changes in the ribo- and deoxyribonucleoside triphosphate pools. In L1210 cells exposed to methotrexate alone, the deoxythymidine 5'-phosphate (dTTP) pools decreased, whereas deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, and deoxycytidine 5'-triphosphate (dCTP) remained relatively constant up to 70% inhibition of growth rate, with dCtP at a constant 112% of controls. The corresponding ribonucleoside triphosphates decreased only slightly. With the combination of methotrexate and thymidine resulting in up to 40% inhibition of growth rate, there was also a decrease in the dTTP pool while the other deoxyribonucleoside triphosphates remained relatively constant, and the corresponding ribonucleoside triphosphates again decreased only slightly. The dCtP pool was reduced to a constant 42% of control comparable to that produced by thymidine alone. With greater than 40% (with thymidine) or 70% (without thymidine) inhibition of growth rate, all pools decreased, but only dTTP was substantially reduced in proportion to the growth rate inhibition caused by methotrexate. The dCtP pool became depleted in spite of the presence of exogenous thymidine. Evaluation of CCRF-CEM cells indicated that inhibition of growth rate and nucleotide pool perturbations by methotrexate were similar to those observed in L1210 cells. However, in the presence of thymidine, inhibition of growth rate appeared related to decreased pools of dCtP, deoxyadenosine 5'-triphosphate, and deoxyguanosine 5'-triphosphate, rather than dTTP as was observed for L1210 cells. Hence, mammalian cells were capable of responding in a differential fashion to pharmacological perturbations, and this capacity may play a role in determining therapeutic selectivity. Since the ribonucleoside triphosphate decreases were slight and relatively uniform during methotrexate-induced perturbations, the deoxyribonucleoside triphosphate pools appear to be more directly related to inhibition of growth rate. The results are consistent with the concept that slight imbalances in the deoxyribonucleoside triphosphate pools dramatically inhibit DNA synthesis, as mediated through their interaction with DNA polymerase.

INTRODUCTION

Growth inhibition by MTX3 is generally attributed to its binding to dihydrofolate reductase (46) resulting in tetrahydrofolate depletion (29) and subsequent cessation of DNA, RNA, and protein synthesis. However, a significant correlation between the oncolytic activity of the drug and this inhibition of dihydrofolate reductase is not apparent (33, 34). Hence, extensive attempts have been made to determine which other parameters might better correlate with MTX inhibition of growth (10, 16–19, 21, 30–34, 39, 41, 42, 48). Definitive correlations have not been elucidated.

Another approach to the problem has been to determine which precursors of DNA, RNA, or protein synthesis are primarily affected during MTX inhibition and which are secondarily affected (32, 45, 49, 50). Protein (and amino acid) synthesis is affected secondary to pyrimidine and purine synthesis (45, 49, 50). The latter 2 have not been consistently differentiated. Wells and Winzler (45), using human leukemia cells, indicated that 1000 nM MTX inhibits formate incorporation into the thymine of DNA more effectively than into purines of RNA. Roberts and Warmath (32), however, using the human leukemia cell culture line, CCRF-CEM, concluded that 50 to 500 nM MTX inhibited glycine incorporation into thymidines and purines in a similar manner. It is of interest that their data with 20 nM MTX showed a slight increase of incorporation of glycine into RNA and a more pronounced increase into DNA. It is possible that the data obtained with 20 nM MTX, a concentration still inhibitory to growth, were not compounded by factors associated with cell death. Thus, a range of noncytotoxic inhibitory concentrations of MTX may be useful for determination of the primary sites of action on nucleic acid synthesis.

The effects of MTX on the growth and viability of some mammalian cell lines can be partially prevented by thymidine alone (up to 7- to 8-fold more MTX is required for the inhibitory effect comparable to that in the absence of thymidine) but not by purines, while for other cell lines, the effects can be prevented by purines but not by thymidine (2, 3, 7, 12, 15, 19, 20, 32, 42). In all cell lines, the addition of both thymidine and a source of purine such as hypoxanthine completely prevents MTX toxicity. The information on the mode of action of MTX from these studies is seemingly conflicting. However, a comparison of MTX effects on nucleotide and folate pools of cell lines showing preferential thymidine or purine prevention of toxicity has not been reported.

Metabolic correlations, precursor incorporations, and metab-

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3 The abbreviations used are: MTX, methotrexate; rNTP, ribonucleoside triphosphate; dNTP, deoxyribonucleoside triphosphate.

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olite reversal studies have yet to lead to a workable hypothesis for predicting MTX action on specific cell types. As a result, marginally successful therapeutic treatments have been empirically derived (1, 6, 7, 35, 51). New methodology may be required to understand and rationally predict the sensitive metabolic interactions in a cell exposed to MTX with or without other effectors. An open steady-state system as considered by Webb (44) and more recently discussed by Grindey et al. (13), involving cell adaptation to new steady-state rates of growth, offers additional and possibly more applicable insights into the nature or pattern of drug actions. This approach could thus lead to the improvement of chemotherapy, particularly combination chemotherapy. Given the high affinity of MTX for dehy- drofolate reductase, the establishment of new steady states is related to (a) the amount and rate of synthesis of enzyme (b) the ratio of extracellular MTX to intracellular exchangeable (free) MTX, as governed by specific transport characteristics (11), and (c) the dissociation of the enzyme-MTX complex (7). Since these parameters also manipulate the nucleotide-syn- thetic pathways, subsequent nucleotide pool alterations may directly determine the final growth rate of cells exposed to particular concentrations of MTX and resultant cytotoxicity.

With logarithmic growth of cells, the transient adaptations to drug perturbations would cease in time as new steady-state growth rates are established. Correlations of these new growth rates with the subsequent stable pool sizes seem likely to lead to new insights into drug effects. No steady-state evaluation of the effects of MTX on rNTP pools has been reported. Hryniuk (20) has described the changes in NTP pools of L5178Y cells exposed to 1000 nM MTX. ATP and GTP pools were greatly reduced. UTP and CTP pools increased initially and then declined in time. Earlier reports on dNTP pool perturbations by MTX have been concerned with a variety of drug concentra-
tions and cell growth conditions other than steady state and have shown varying effects on all 4 DNA precursors (9, 39, 41–43). In previous reports from our laboratory, addition of deoxynribonucleosides to medium has given rise to reduced steady-state growth rates of L1210 cells where slight changes, not limitations, in the dNTP pools were correlated with substantial inhibitions of growth rate (25). These results have suggested that imbalances of the dNTP pools and the possible effects they might have on DNA polymerase activity could be responsible for inhibition of DNA synthesis and subsequent inhibition of growth rate (25). The present study is an extension of this premise to drug effects on nucleotide pools of leukemia cells in steady-state growth. Evaluated then are the effects of 5 to 25 nM MTX on (a) growth rate, (b) the rNTP and dNTP pools, and (c) the modification of the growth rate and pools by addition to the medium of an amount of thymidine which maximally reverses MTX inhibition without itself inhibiting growth (12). While the methodology of this paper is based on previously described procedures (25), several important improve-
ments were required for accurate and reproducible quantitation of the small changes in nucleotide pools which were observed. A more precise description of the methods is thus included.

MATERIALS AND METHODS

Chemicals. MTX was purchased from Lederle Laboratories, Pearl River, N. Y., while nonlabeled nucleotides and thymidine were purchased from Sigma Chemical Co., St. Louis, Mo., and P-L Biochemicals, Inc., Milwaukee, Wis. [3H]dATP (14 Ci/ mmol), [3H]dTTP (15 Ci/mmol), [3H]dGTP (19 Ci/mmol), and [3H]dCTP (18 Ci/mmol) were from Schwarz-Mann, Orange-
burg, N. Y. Lyophilized noncovcalent double-stranded copoly-
mer of deoxypolyadenylate and deoxypolythymidylate and non-
covalent double-stranded copolymer of deoxypolyinosinate and deoxypolyctydylate-sodium, were purchased from P-L Biochemicals, and for use were suspended at 450 μg/ml in 0.1 M NaCl. The Micrococcus luteus DNA polymerase, (specific activity, 1336 units/mg protein) was purchased from Miles-Pentex Laboratories, Elkhart, Ind. Fresh stock dNTP solutions were made every 6 to 8 weeks. All stock solutions and materials were stored at −20°C.

Cell Culture. Both the murine leukemia L1210 cells (14) and the human leukemia CCRF-CEM cells (8, 30, 31) were grown at 37°C in stationary suspension cultures in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% dialyzed fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.) and 20 μM morpholinopan-
anesulfonic acid, pH 7.3 (Sigma Chemical Co.). All experiments were conducted using 250-ml volumes in 500-ml Ros-
well Park Memorial Institute bottles. Neither the morpholinopan-
anesulfonic acid buffer nor the culture volume affected opti-
mal cell growth kinetics. Cell numbers were determined using a model ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.).

The L1210 cells grew exponentially from a 0.25 × 10⁶/ml inoculum to 3 to 4 × 10⁶ cells/ml in the bottles, with about a 12-hr doubling time (see Chart 1). Results of preliminary ex-
periments suggested the use of cell densities less than 2.5 × 10⁶ cells/ml as well as the use of an atmosphere of 5% CO₂ for optimal cellular concentrations of the nucleotide pools. Using these conditions, which allow for 10-fold growth of control L1210 cultures within 38 hr, the 4 rNTP pools were maintained at their highest level and the ribonucleoside mono-
diphosphates were at their lowest. Further, the dNTP pools were also maintained at their highest levels with cell densities between the inoculum and 2.5 × 10⁶/ml. A change in cell density from 2.5 to 5 × 10⁶/ml, for instance, showed de-
creases in the ATP pool from 2400 to 600 pmol/10⁶ cells. Hence, large volumes of a stock culture of L1210 in 500-ml Roswell Park Memorial Institute bottles, loosely capped in a 5% CO₂ incubator, were fed with fresh medium daily, maintain-
ing a logarithmically growing low-density cell culture with suf-
ficient total numbers of cells for experimentation. Dilution of cells from the stock culture to the experimental concentration was thus small and minimized alterations in nucleotide pools and macromolecular synthesis caused by the fresh medium and handling. These alterations continued for up to 6 hr after onset of an experiment (dilution) before the establishment of the steady, high-triphosphate, low-monophosphate-, and low-
diphosphate nucleotide conditions; the length of time to reach steady state increased with the stock cell density. The nucleo-
tide pool sizes, therefore, have provided a slightly more sensi-
tive parameter than growth kinetics to define cell density and time limits for steady-state conditions. The establishment of new steady-state growth kinetics (stable doubling times) by drug-treated cells was achieved in all cases before harvesting and within the steady-state limits set by the controls. Harvesting of the cells for nucleotide extraction was done routinely at 29 hr of growth, a cell density of 1.5 to 1.8 × 10⁵/ml for the controls.
The CCRF-CEM cells grew exponentially from the 0.3 x 10^5 cells/ml inoculum to 6 x 10^5 cells/ml with about a 20-hr doubling time (Chart 1) and were harvested for extraction at 48 hr. Both the L1210 and CCRF-CEM cell culture lines were routinely checked for Mycoplasma contamination by Microbiological Associates, Walkersville, Md., with negative results.

**Preparation of Cell Extracts.** Control and drug-treated cells were harvested by cooling to 4°C; 2.25 x 10^5 cells (in all cases) in a 500-ml Erlenmeyer flask were swirled in a dry ice-ethanol bath for 15 to 30 sec. The cells were centrifuged at 1500 to 2000 rpm for 15 min at 4°C in a Beckman Model J-21 centrifuge (using a JS 7.5 swinging bucket rotor). The medium was discarded, and any amount remaining inside the bottles was carefully removed with Kimwipes. Washing the cells was thus averted, preventing a substantial loss of nucleotides (e.g., 40% when washed 4 times without a glass rod). The acid mixtures were transferred to Biovials (Beckman), another 400 μl of 1 M HClO4 were used as a wash, and the wash was added to the Biovials. After 15 min, the vials were centrifuged at 2500 rpm for 7 min at 4°C, and the supernatants were transferred to clean vials. Another 200 μl of 1 M HClO4 were added to the precipitate for a second extraction and the extracts were combined. To the supernatants was added 40 μl of 0.5 M Tris-HCl, pH 8.3, and then 125 μl of 8 N KOH ±1- to 5-μl increments of 1 M HClO4 or 8 N KOH, to bring the pH to between 8.0 and 8.3. The KClO4 precipitate was removed by centrifugation, and the supernatants were stored at −20°C and assayed with 2 weeks for nucleotide pools.

**Determination of Ribonucleotide Pools.** Separation of the acid-soluble ribonucleoside mono-, di-, and triphosphates, was achieved using a DuPont 830 high-pressure liquid chromatograph as described by Rustum (36). The mobile phase on the Permpahse ABX column (E. I. duPont de Nemours & Co., Wilmington, Del.) was a linear gradient of 2 mM KH2PO4, pH 3.0, to a 500 mM KH2PO4 solution, pH 4.6. The rate of addition of the high-phosphate buffer was 3%/min to a final composition of 63%. Quantitation was done by means of peak height measurements. Chart 2 shows the graphic profiles of a standard mixture of 17 ribonucleotides at 2 different concentrations. A flow rate of 0.35 to 0.37 ml/min at an air pressure of between 275 and 300 psig and periodic washing of the column with 100% of the high-molarity KH2PO4 for 5 min at 1 ml/min were essential for the precise reproducibility of the elution profile. The lower limit of sensitivity at the sensitivity of all of the ribonucleotides was routinely 2.5 pmol in a 10-μl injection (0.25 μm), with the availability of a further 10-fold sensitivity gain in the instrument detection system. Control tissue culture cells showed nucleotide levels between 30 and 750 pmol/10-μl sample (3 to 75 μm in 2.5 x 10^5 cell equivalents/ml) and hence were readily analyzable.

**dNTP Assay.** The assay of the acid-soluble dNTP pools was based on that originated by Solter and Handschumacher (40). However, synthetic heteropolymers, deoxyadenylate-deoxythymidyde and deoxyninosinate-deoxycytidylate afforded greater sensitivity than did calf thymus DNA template, and the M. luteus DNA polymerase was used in preference to the Escherichia coli enzyme to reduce nuclease activity. In comparison to previously reported reaction conditions using synthetic polymers and the E. coli enzyme (24, 38), the use of M. luteus enzyme extended the lower sensitivity from 0.5 to 0.2 ± 0.1 (S.D.) pmol (with a large decrease in background) and increased the higher sensitivity from 7 to 40 pmol.

Reaction conditions follow: 0.1 μg double-stranded alternating copolymer of deoxyadenylate and deoxythymidyde per 200-μl reaction volume was found to be optimal. More polymer increased background counts excessively. The M. luteus DNA polymerase was added at 1.5 units for the dATP, dTTP, and dGTP measurements, but 2 units were required for complete dCTP incorporation. Reaction time was 25 min. Time course reactions revealed nuclease activity only on the deoxyadenylate-deoxythymidyde product and only after a 25-min incubation. Complete incorporation was reached between 22
and 25 min for each system. Reactions were buffered with 11 μmol (55 mM) Tris-HCl, pH 8.3. Each reaction contained 0.44 μmol (2.2 mM) MgCl₂. A minimum of 1 mM MgCl₂ was required. Greater than 5 mM MgCl₂ decreased dGTP and dCTP incorporation of both standards and cell extracts. Each reaction system also contained 0 to 40 pmol (0 to 0.2 μM) of the limiting substrates (standard or 0.025 to 0.1 × 10⁶ cell equivalents), and 150 pmol (0.75 μM) of the excess substrate, including labeled compound at 0.665 μCl [³H]dNTP, (specific activity, 14 to 19 Ci/mmol). In addition, the reaction contained 0.5 μmol (2.5 mM) 2-mercaptoethanol or dithiothreitol, and distilled water were then added to the volume. Under these conditions, the incorporation of radioactivity into polymer was 700 to 1200 cpm/pmol dNTP.

The limiting dNTP was added in 100-μl aliquots to 12-× 75-mm polyethylene tubes in an ice-water bath, either as the known standard or as the neutralized cellular extract, both diluted appropriately in saturated KClO₄ solution, pH 8.3. Premixes containing the other reaction components but excluding the limiting dNTP were kept below 2-ml volumes to assure low background counts. Enzyme was added to the premixes, and 100 μl of the appropriate premixes were added to the complementary limiting deoxyribonucleotides in the assay tubes to start the reactions. The tubes were shaken briefly; then set in a 37°C water bath. Reactions were terminated by transferring the tubes to an ice-water bath. To separate the macromolecular product from the free-labeled dNTP, the tubes were sampled immediately by spotting 100 μl on Whatman No. 3MM paper discs, 2.3 cm in diameter. The discs were placed in ice-cold 5% trichloroacetic acid-1% sodium pyrophosphate (about 20 ml/disc), where they remained for at least 30 min. The discs were then washed in 5% trichloroacetic acid-1% sodium pyrophosphate, ethanol, and ether by the method of Lowe and Grindey (25). They were then dried in an oven at 65°C for 15 min, placed in glass scintillation vials containing 5 ml Omnifluor (New England Nuclear, Boston, Mass.), and counted in a Mark III liquid scintillation spectrometer (Searle, Des Plaines, Ill.).

At least 4 concentrations of standards in the range of the unknown were run with each new reaction premix. The unknown extracts were run in 3 dilutions to assure linear incorporation of radioactivity into polymer. Further, when known amounts of standard dNTP were added to extracts to evaluate assay recovery, the effects were additive. Inhibition by rNTP pools, incorporation of rNTP pools, and extraneous incorporation by deoxyribonucleoside mono- and diphosphates were not effectors in the dNTP measurements. All L1210 results are the mean of duplicate or triplicate growth experiments, each containing 5 or 6 different growth rate conditions; each growth rate condition was assayed in duplicate for rNTP pools and in triplicate for dNTP pools.

**RESULTS**

Growth kinetics of L1210 and CCRF-CEM cell suspensions have been previously described by Moran (28) for MTX inhibition in the presence or absence of thymidine using a small-volume (2-ml) system. Chart 1 shows comparable MTX inhibition kinetics for both cell lines, with and without thymidine present, using the large-volume (250-ml) system described in "Materials and Methods." Exposure of L1210 cells to MTX showed a sharp dose-response relationship ranging from no inhibition of growth rate at 5 nM MTX to 90% inhibition of growth rate at 25 nM MTX. The highest concentration of thymidine which is noninhibitory to the growth of L1210 cells in culture is 5.6 μM (12). Addition of this concentration of thymidine to L1210 cells simultaneously exposed to MTX delayed the inhibition of growth, and the cells established increased steady-state rates compared to those expressed in the presence of MTX alone. The CCRF-CEM cells also established reduced steady-state rates of growth in response to MTX and maintained these rates of growth for up to 70 hr. Since addition of 5.6 μM thymidine to CCRF-CEM cells resulted in some growth inhibition, lower concentrations of thymidine were utilized. Although still slightly inhibitory to CCRF-CEM cells, 2.8 μM thymidine modified MTX inhibition of growth rate in a manner similar to the effect of 5.6 μM thymidine on L1210 cells (Chart 1B).

Ribonucleotide pool measurements were used to supplement cell growth kinetics in defining limits of cell density and length of incubation for steady-state growth. In particular, the sustained maximum levels of rNTP pools were used to establish reproducibility of conditions for nucleotide and nucleic acid synthesis, thus avoiding the transient changes commensurate with both experimental manipulation and drug perturbation. Since the cellular extracts showed only small amounts of monophosphates with the growth conditions used and since nucleosides and bases eluted with CMP and AMP, the monophosphates were not measured. In L1210 cells, diphosphate measurements generally showed slight decreases, 25 to 50% of controls, between 70 and 90% inhibition of growth rate, while they were generally increased by 25 to 50% above controls at less inhibitory effects of the combinations of MTX and thymidine. Thymidine alone increased the diphosphates.
uniformly 35 to 60% above controls. In the CCRF-CEM cells, the diphosphates showed a gradual decrease following exposure to MTX similar to the rNTP pool responses described in Table 1.

Table 1 shows the nucleoside triphosphate pools of CCRF-CEM cells resulting from MTX inhibition with and without thymidine in a study ranging from 38 to 90% inhibition of growth rate. For the 2 MTX concentrations tested, the addition of 2.8 μM thymidine substantially increased the rNTP and dNTP pools in parallel with the reduction in growth rate inhibition. The rNTP pools showed a gradual decrease with the decreased growth rate with ATP most affected. Thymidine alone uniformly increased the rNTP pools 13 to 18% above controls.

Whereas the rNTP pools of CCRF-CEM cells gradually and uniformly decreased with MTX inhibition of growth rate, the dNTP pool responses were quite variable (Chart 3). Thymidine alone produced an 8.6% inhibition of growth rate under conditions where only dTTP differed significantly from control values and was increased 300%. At 38% inhibition in the presence of MTX and thymidine, the dTTP pool was still 3-fold higher than control, the dATP and dGTP pools were only slightly reduced whereas the dCTP pool decreased to 27% of control (Chart 3). By 71% inhibition of growth rate in the presence of this combination, the dTTP concentration was still uniformly decreased with MTX inhibition of growth rate, the dATP and dGTP pools were now also substantially reduced. These reductions in dATP and dGTP were directly parallel to alterations in the ATP concentration as well (Table 1).

Charts 4 to 7 show the changes in the rNTP and dNTP pools with changes in steady-state growth rate resulting from MTX inhibition of L1210 cells with and without thymidine in the medium. Charts 4 and 5 depict the responses of rNTP and dNTP pools, respectively, in cells exposed continuously to various concentrations of MTX. Each rNTP pool is eventually decreased to between 50 and 80% of controls.

The highest concentration of MTX which did not inhibit growth rate (5 nM) increased both dCTP and dTTP above controls while not affecting dATP and dGTP pools. With increasing concentrations of MTX and subsequent increasing inhibition of growth rate, the dTTP pool decreased continuously, dropping below control levels only after 35% inhibition of growth rate. Simultaneously, the other dNTP pools remained relatively constant, or decreased very slowly up to 70% inhibition of growth rate, with dCTP at a constant 112% of controls. From 70 to 90% inhibition of growth rate by MTX, all 4 pools decreased, dTTP being most affected, dropping to 16% of control.

Chart 6 depicts the rNTP pool and growth rate responses relative to controls in cells continuously exposed to various concentrations of MTX and 5.6 μM thymidine. This highest concentration of thymidine, which did not inhibit growth by itself, caused the rNTP pools to decrease 5 to 10% relative to controls. These decreases were not observed when the cells were exposed to, but not inhibited by, thymidine and 8 nM MTX. With increasing concentrations of MTX and subsequent

Table 1

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>% of inhibition of growth rate</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>UTP</td>
</tr>
<tr>
<td>2.8 μM dThd</td>
<td>8.6</td>
<td>117.8</td>
</tr>
<tr>
<td>14 nM MTX + 2.8 μM dThd</td>
<td>37.8</td>
<td>82.2</td>
</tr>
<tr>
<td>25 nM MTX + 2.8 μM dThd</td>
<td>71.1</td>
<td>49.1</td>
</tr>
<tr>
<td>25 nM MTX</td>
<td>78.4</td>
<td>35.4</td>
</tr>
</tbody>
</table>

* pmol rNTP and dNTP per 10^6 cells for control cultures: ATP, 3457; UTP, 2163; GTP, 1155; CTP, 667; dATP, 28.8; dTTP, 57.3; dGTP, 27.7; and dCTP, 16.8.

** dThd, thymidine.

*ND, not detectable.

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Steady-State Nucleotide Pool Responses to MTX-Thymidine

Effect of MTX or MTX plus thymidine on CCPF-CEM cells in culture

Growth rates were determined by comparing inhibited and control slopes between 25 and 70 hr of incubation (see Chart 1B). All results are the average of 2 separate experiments.

Table 1

Effect of MTX or MTX plus thymidine on CCPF-CEM cells in culture

Growth rates were determined by comparing inhibited and control slopes between 25 and 70 hr of incubation (see Chart 1B). All results are the average of 2 separate experiments.
increasing inhibition of growth, all 4 rNTP pools decreased very slowly up to the 40% inhibitory level, similar to their responses in cells exposed to MTX alone. However, with growth rate inhibitions between 40 and 85% of controls, the presence of thymidine resulted in rNTP pool decreases 15 to 30% more than those resulting from exposure of cells to MTX alone. Chart 7 shows the dNTP pool and growth rate responses relative to controls in L1210 cells exposed to various concentrations of MTX and 5.6 μM thymidine. Combinations of MTX and thymidine further increased dTTP to 218% of control, kept dCTP at 42% of control, and did not alter dATP and dGTP. With increasing concentrations of MTX in the presence of 5.6 μM thymidine, the dTTP pool decreased continuously, linearly with the subsequent inhibition of growth rate. The dTTP pool, above the control value up to 50% inhibition of growth rate, decreased to 42% of control by 84% inhibition of growth rate. At the same time, this coaddition of thymidine, with increasing concentrations of MTX, held the dCTP constant at 42% of control up to 60% inhibition of growth rate. Between 60 and 84% growth rate inhibition, the dCTP pool decreased only slightly to 30% of the control. The purine dNTP pools, dATP and dGTP, remained unchanged to 40% inhibition of growth rate and then decreased to 47 and 66% of controls, respectively, by 84% inhibition of growth rate.

DISCUSSION

Open steady-state systems have been considered in detail by Webb (44), who made a major contribution to our understanding of the action of antimetabolites in multiple enzyme networks. Such a network is in a true steady state when the concentrations of all intermediates and the rates of all reactions remain constant with time. Mammalian cells in culture during stable exponential growth, in the absence of physiological or pharmacological stress, represent an excellent approximation of an open steady-state system. Under such conditions, nutrients enter while waste products leave, and essentially constant levels of intermediates and enzymes are maintained. In the present study, the establishment of such a steady state under the culture conditions for control and drug-treated cells was characterized by stable ribonucleotide pools, as well as exponential growth. (See 'Materials and Methods'). Physiological or pharmacological stress will cause transient departures from the steady state during the course of adaptation to the new characteristics of a modified or inhibited steady-state system. The inability to establish a new steady state under such stress would eventually be lethal to the cell (13, 23, 44, 47). The action of inhibitors must therefore be considered with respect to the steady state; it can be expected that their effects will be dependent on the overall characteristics of...
the enzymes and regulatory metabolites that define the initial
steady-state network (4, 5, 11, 13, 23, 26, 27, 37, 44, 47).

In this study, growth-inhibitory concentrations of MTX and a
noninhibitory concentration of thymidine have been used to
evaluate the relationship between steady-state growth rate and
nucleoside triphosphate pools. Table 1 and Charts 3 to 7 show
the patterns of these pool size adaptations to MTX inhibition in
CCRF-CEM and L1210 cells, respectively. MTX effects were
manifested in L1210 cells by an initially elevated dTTP pool
under conditions which did not inhibit growth followed by a
decrease in dTTP with reduction in the growth rate (Chart 5).
The other dNTP pools remained relatively constant up to 70%
inhibition of growth rate. The slight decreases in the corre-
sponding rNTP pools appeared unrelated to these changes in
dNTP pools (Chart 4). The addition of thymidine to the medium
substantially altered the initial effects of MTX by decreasing
the pool of dCTP (Chart 7). However, the inhibition of growth
by increasing concentrations of MTX were again correlated
only with a reduction in dTTP (Chart 7). The changing dTTP
pool and an altered dCTP pool are thus the only apparent
disturbances caused by MTX alone or in the presence of
thymidine under conditions of substantial growth rate inhibition.

The pool size patterns in Charts 5 and 7 are consistent with
the concept that there is a direct correlation between the rate
of DNA synthesis (as determined by growth rate inhibition) and
imbalanced DNA precursors. Reduction in the dTTP pool ap-
pears to be correlated with increasing inhibition of growth rate
for L1210 cells. This observation is consistent with current
concepts for effects of MTX alone but is completely unexpected
in the presence of thymidine. Based on metabolite reversal
studies (2, 3, 7, 12, 15, 19, 20, 22, 32, 42), the effects of MTX
in the presence of thymidine were thought to result exclusively
from a purine deprivation. As shown in Chart 7, however, there
is an excellent correlation between the size of the dTTP pool
and growth rate inhibition while the purine nucleotide pools are
not substantially reduced. As discussed below, this unexpected
finding must be a function of the complicated regulatory prop-
erties involved in DNA metabolism (Chart 8).

Mathematical models of DNA metabolism, which included
the complicated regulatory properties summarized in Chart 8,
were previously constructed to evaluate the interaction be-
tween various combinations of antimetabolites in this system
(13, 23, 47). Such models, based on steady-state assumptions,
led to several unexpected predictions: (a) that the presence of
a single inhibitor such as MTX in a regulated network caused
pronounced changes in the concentration of all the intermedi-
ate metabolites in the network; thus, the effects of a perturba-
tion in one region will not be localized but rather will be
manifested throughout the entire system; (b) that such feed-
back regulation could lead to perplexing experimental results;
e.g., extensive inhibition of a single pathway within such a
network could result in an increased, decreased, or even
unchanged pool of the product of that pathway; (c) that the
changes in the pools of metabolic intermediates in the network
were not intuitively obvious and could not be readily predicted;
(d) that concentrations of intermediates need not change
smoothly from one state to the other, but may overshoot,
transiently move in a direction opposite to the final concentra-
tion, or undergo a damped oscillation; thus, attempts to deduce
the effect of an inhibitor on such a system by consideration of
initial or non-steady-state effects may be misleading; and (e)
that the resultant perturbations that occur in the system may
vary dramatically from one condition to another, dependent on
the characteristics of the initial steady state; thus, drug-induced
perturbations in metabolite pools in mammalian cells may vary
substantially from one cell to another.

The unexpected changes in metabolite pools induced by
MTX observed in this study confirm many of the predictions
generated by the mathematical models. For example, the ad-
dition of thymidine to the culture medium induces new steady
states for both CCRF-CEM and L1210 cells as evidenced by
changes in the deoxynucleoside triphosphate pools (Table 1;
Chart 7). As shown in Charts 3 and 7, the effects of MTX in the
presence of thymidine on deoxynucleotide pools are quite
different in CCRF-CEM cells from those observed in L1210
cells. In CCRF-CEM cells, dTTP pools remain elevated in the
presence of thymidine (Table 1) while dCTP is dramatically
reduced. The pools of dATP and dGTP also fall at the higher
levels of inhibition. While no single deoxynucleotide pool can
be related to the observed inhibition, the average decrease in
dCTP, dATP, and dGTP appears sufficient to account for the
reduced growth rate effects. For L1210 cells (Chart 7), only
the dTTP pool is decreased in relation to the observed growth
rate inhibition.

Thus, markedly different perturbations in deoxynucleoside
triphasate pools can be induced in different cell lines under
identical conditions of pharmacological insult. As indicated by
the mathematical models, such unexpected and variable re-
sponses can be attributed to differences in overall regulatory
characteristics as determined by the intracellular concentra-
tions of key enzymes and regulatory metabolites in various
types of cells. Interestingly, the addition of a normal metabolite
such as thymidine to the system can induce changes in these
regulatory properties and substantially alter drug effects in an
intuitively unpredictable fashion.

Previous studies have also shown varying effects of MTX on

Steady-State Nucleotide Pool Responses to MTX-Thymidine

Chart 8. Summary of metabolic regulation involved in DNA synthesis. — reaction pathways; — — feedback activation; • • • • feedback inhibition. This summary is based on the characteristics reported for ribonucleotide reductase (5, 27) and deoxycytidylate deaminase (26, 37). The information on salvage pathways has been omitted.
REFERENCES

Evaluation of Ribonucleoside and Deoxyribonucleoside Triphosphate Pools in Cultured Leukemia Cells during Exposure to Methotrexate or Methotrexate Plus Thymidine

James J. Kinahan, Maureen Otten and Gerald B. Grindey


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