Selective Inhibition of Pyrimidine Biosynthesis and Effect on Proliferative Growth of Colonic Cancer Cells

Kenneth K. Tsuboi, Henry N. Edmunds, and Linda K. Kwong

Department of Pediatrics, Section of Developmental Medicine, Stanford University, Stanford, California 94305

SUMMARY

A highly selective inhibition of de novo pyrimidine synthesis in the intact cell has been demonstrated by the action of N-(phosphonacetyl)-L-aspartate (PALA), a transition-state analog inhibitor of the reaction catalyzed by aspartate transcarbamylase. The effect of pyrimidine deprivation induced by this agent on the viability and survival of human normal (WI-38) and colonic cancer cells (HT-29) was examined. The PALA-treated, pyrimidine-deprived cells failed to grow but demonstrated a normal rate of glucose utilization with impaired glycogen synthesis. Pyrimidine deprivation and lack of cell growth were maintained long after PALA removal. Growth inhibition of HT-29 cells by PALA was found to reflect an apparent steady state between newly formed and dying cells induced by limited pyrimidine availability. The highly selective nature of PALA action was confirmed by the ability of an exogenous source of pyrimidine to restore the normal growth pattern of the cell. Significant antitumor activity by PALA was found against a transplantable colonic tumor (line 26) carried in mice.

INTRODUCTION

Potent and selective inhibition of mammalian aspartate transcarbamylase by an analog of the activated complex of the reaction catalyzed by this enzyme has been reported (5, 15). The analog, PALA, has been shown to inhibit proliferative growth of mammalian cells (15, 19) and to exhibit considerable antitumor activity against certain transplantable tumors in mice (9).

In the present study, inhibition of de novo pyrimidine synthesis by PALA has been examined in relation to the growth and viability of normal (WI-38) and colonic cancer (HT-29) cells of human origin. PALA was found to be a highly selective and intense inhibitor, resulting in little measurable de novo synthesis of pyrimidine both during and after treatment of the cells with this agent. Short- and long-term effects of cell pyrimidine deficiency induced by this agent on the viability and survival of human normal (WI-38) and colonic cancer cells (HT-29) was examined. The PALA-treated, pyrimidine-deprived cells failed to grow but demonstrated a normal rate of glucose utilization with impaired glycogen synthesis. Pyrimidine deprivation and lack of cell growth were maintained long after PALA removal. Growth inhibition of HT-29 cells by PALA was found to reflect an apparent steady state between newly formed and dying cells induced by limited pyrimidine availability. The highly selective nature of PALA action was confirmed by the ability of an exogenous source of pyrimidine to restore the normal growth pattern of the cell. Significant antitumor activity by PALA was found against a transplantable colonic tumor (line 26) carried in mice.

MATERIALS AND METHODS

Materials. [14C]Carbamylphosphate (7.4 mCi/mmmole), Ba[14C]CO3 (60 mCi/mmmole), and (methyl-3H)thymidine (86 mCi/mmmole) were obtained from New England Nuclear, Boston, Mass. Na14CO3 was prepared from Ba14CO3 as purified previously (7), and [14C]carbamylphosphate was purified as described by Adair and Jones (1). Purified carbamylphosphate was stored in small aliquots at -70°C. Cytosine arabinoside, hexokinase, glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.), 5-fluorouracil, and dilithium carbamylphosphate (Calbiochem, LaJolla, Calif.) were obtained from the indicated sources. PALA and (3H)PALA were prepared as described by Swyryd et al. (15).

Cell Lines and Culture. HT-29, a human colonic epithelial cancer cell line (3), was obtained from Dr. J. Fogh. WI-38, a human diploid fibroblast cell line originating from normal fetal lung, was obtained from Dr. L. Hayflick. The cells were subcultured and grown in Eagle's minimal essential medium supplemented with gentamicin at 50 μg/ml, vitamins, and amino acids at twice the normal concentrations and dialyzed or nondialyzed fetal bovine serum at 5% for HT-29 and 10% for WI-38 cells. Cells were subcultured twice weekly and also were maintained as frozen stocks. Cells were grown for experimental use in plastic T-75 flasks, 60- and 35-mm Petri dishes, and cluster dishes containing 24 wells of 15-mm diameter. Growth medium was changed every 3 days unless specified otherwise.

Tumor Transplant. Line 26, a transplantable colonic tumor (undifferentiated grade IV carcinoma) induced in BALB/c mice by 1,2-dimethylhydrazine, was obtained from Dr. T. H. Corbett (for tumor details see Ref. 2). The tumor was maintained by serial passage at 2- to 3-week intervals in BALB/c mice and also as frozen stock. The mice were fed a standard laboratory chow (Berkeley diet), except a synthetic pyrimidine-free diet (63% sucrose, 18% casein, 5% vegetable oil, 5% fiber, vitamins, and balanced salts) was substituted during the period of experimental PALA administration. Mice were inoculated s.c. with 0.1 ml of a 10% finely minced suspension of the tumor in Hanks' medium.

Incorporation of [14C]Carbonate into Pyrimidines. Cells grown in T-75 culture flasks were incubated in the stopped flask for specified periods in Hanks' medium modified to contain 10 mM N-tris(hydroxymethyl)methyl-2-amino-
ethanesulfonic acid buffer, pH 7.4, and 1.0 mM sodium [14C]carbonate (50 µCi). After incubation the cells were washed with cold Hanks' medium, scraped, transferred, and centrifuged in capped 1.5-ml plastic tubes. The cells were air dried after extraction with 1.0 ml absolute ethanol containing 10 mM potassium acetate. To the dried cells were added 200 µl of 70% perchloric acid followed by 1 hr heating at 100° to effect release of free pyrimidines and purines from acid-soluble nucleotides and nucleic acids. The procedure was adapted from an original application for quantitative base isolations from nucleic acids (18) in which complete hydrolysis of bases has been shown to be achieved with minimal deamidation of cytosine to uracil resulting. After addition of 200 µl H2O to the hydrolysate, the total sample (less 10-µl aliquot for protein) was applied in 50-µl aliquots to a 9.5- x 22.5-inch sheet of Whatman No. 3 MM paper and chromatographed (placed in Chromatocab immediately) for 24 hr in isopropyl alcohol/HCl/H2O (65/17/18). The solvent resolves cleanly guanine, adenine, cytosine, uracil, and thymine in ascending order from their mixture (18). After elution (0.1 N HCl) amounts of each base were determined by UV absorption and purified further by adsorption and elution from charcoal (17) and rechromatographed for 24 hr in H2O-saturated butyl alcohol containing excess NH4HCO3 (17). Radiochemical purity was established by coincidence of radioactivity (determined by radioautography) and UV absorption of the final isolated pyrimidine (and purine) deposits (17) as illustrated in Fig. 1.

Cell Glycogen Determinations. Cells were washed free of medium and transferred, after scraping in 1.0 ml of 0.5 M perchloric acid in 70% ethanol, to 1.5-ml capped plastic centrifuge tubes. After centrifugation, the packed cells were extracted with 1.0 ml absolute ethanol containing 10 mM potassium acetate and air dried. To the dried cells were added 150 µl of 0.6 N HCl, and the capped tubes were heated 2 hr at 100°. After neutralization with NaOH, aliquots were removed for protein and glucose determination.

Miscellaneous Methods. Aspartate transcarbamylase assays were as described by Swyryd et al. (15). Glucose was determined by enzyme-coupled stoichiometric reduction of NADP, which was measured fluorimetrically (11). Cell extracts prepared by 20-min hydrolysis at 75° in 0.5 M perchloric acid were measured for DNA (4) and RNA (14) with modification of the published procedures to reduce final color volumes to 1 ml. Application of these methods to cell cultures has been described previously (16). Protein was determined by the method of Lowry et al. (12). Cell growth rate was determined by protein and DNA accumulation and in certain cases by absorbance at 260 nm of a solubilized solution of the cells prepared by sonic extraction in 1.0 M NaOH (15).

RESULTS

Inhibition of Pyrimidine Synthesis. In Chart 1 are shown inhibition of aspartate transcarbamylase, a component enzyme of the pyrimidine-biosynthetic pathway, of HT-29 (human colon cancer), and of WI-38 (human diploid fibroblasts) cells by PALA, an analog of the activated complex of the enzyme-catalyzed reaction (15). Inhibition by PALA was competitive with carbamylphosphate (5) and equally intense on the aspartate transcarbamylases of both HT-29 and WI-38 cells. Kinetic constants derived from the plotted data yielded very similar apparent Km and Kf values for both enzymes, with Kf of 1.0 to 1.4 x 10^-8 M for carbamylphosphate and Kf of 0.85 to 1.1 x 10^-14 M for PALA. Relative aspartate transcarbamylase activities of HT-29 and WI-38 cells were Vmax = 3.7 and 2.3 nmoles carbamylphosphate conversion per min mg protein, respectively.

The effect of PALA, as a selective and intense inhibitor of aspartate transcarbamylase, on de novo pyrimidine nucleotide synthesis in intact HT-29 cells is shown in Table 1. In these experiments, de novo synthesis of pyrimidine was determined by measuring incorporation of [14C]CO2 into total cell pyrimidines. Isolation of the free pyrimidine bases cytosine, thymine, and uracil followed their hydrolytic release from total cellular nucleotide and nucleic acids treated as a common pool. Marked inhibition of pyrimidine synthesis was induced in HT-29 cells by PALA at 1 x 10^-3 M. Inhibition was apparent within 2 hr and near maximal after 4 hr. [14CO2] incorporation into cell pyrimidines was 2 to 4% and 60% of control values after maintaining cells for 24 hr in the presence of 1 x 10^-3 and 1 x 10^-4 PALA, respectively. Inhibition of pyrimidine synthesis in HT-29 cells was also demonstrable by uridine alone (6) or in the combined presence of PALA. Inhibition by uridine was evident within 2 hr at 1 x 10^-4 M and near maximal at 1 x 10^-3 M. Labeled CO2 was incorporated only into uracil after 30 min incubation (see Experiment 2) with further distribution of the label into cytosine and thymine evident only on extending the incubation period with labeled CO2 to 2 hr (see Experiment 1).

Sustained Inhibition of Pyrimidine Synthesis. Inhibition of pyrimidine biosynthesis in HT-29 cells induced in the presence of PALA was found to be maintained even after removal of the inhibiting agent from the culture medium as...
shown in the experiment summarized in Table 2. HT-29 cells
maintained in the presence of 1 mM PALA or 24 hr (and
longer) showed little incorporation of $^{14}$CO$_2$ into pyrimidine.
Inhibition of pyrimidine synthesis by PALA resulted in an
accompanying inhibition of de novo purine synthesis as
evidenced by a lack of $^{14}$CO$_2$ incorporation into cell purines

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
</table>

**Inhibition of pyrimidine synthesis in HT-29 cells by PALA and uridine**

HT-29 cells in T-75 flasks were incubated for the indicated periods with PALA and/or uridine at specified concentrations added to the growth medium. $^{14}$CO$_2$ incorporation into pyrimidines was subsequently determined as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Additions (mM)</th>
<th>Cytosine</th>
<th>Thymine</th>
<th>Uricil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5,500</td>
<td>8,300</td>
<td>77,000</td>
</tr>
<tr>
<td>PALA, $1 \times 10^{-4}$ (24)</td>
<td>2,000</td>
<td>2,200</td>
<td>47,000</td>
</tr>
<tr>
<td>PALA, $1 \times 10^{-3}$ (24)</td>
<td>360</td>
<td>250</td>
<td>3,000</td>
</tr>
<tr>
<td>PALA, $1 \times 10^{-3}$ + uridine,</td>
<td>200</td>
<td>970</td>
<td>1,400</td>
</tr>
<tr>
<td>$1 \times 10^{-3}$ (24)</td>
<td></td>
<td></td>
<td>3,200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PALA, $1 \times 10^{-3}$ (2)</td>
<td>6,100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PALA, $1 \times 10^{-3}$ (4)</td>
<td>2,300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PALA, $1 \times 10^{-3}$ (24)</td>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urd.</td>
<td>11,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urd. $1 \times 10^{-3}$ (2)</td>
<td>6,900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urd. $1 \times 10^{-3}$ (2)</td>
<td>6,200</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Pyrimidines were isolated in each case from 5 to 7 mg cell protein. Cell pyrimidine contents in µmoles per mg protein were:
  - Cytosine: 0.10 ± 0.02
  - Thymine: 0.06 ± 0.02
  - Uricil: 0.08 ± 0.02

* Numbers in parentheses, time (hr).

Inhibition of Pyrimidine Synthesis and Effect on Proliferative Growth of HT-29 and WI-38 Cells. The effect of PALA, added to growth medium at concentrations inducing inhibition of pyrimidine synthesis (as measured by labeled CO$_2$ incorporation) on proliferative growth of HT-29 and WI-38 cells is shown in Chart 2. In these experiments, PALA was added to growing cell cultures and maintained over a 6-day period at 0.1 and 1.0 mM concentrations. The cells were maintained an additional 6 days in culture in the absence of PALA. Proliferative growth was measured in these experiments on the basis of protein, DNA, and RNA accumulation, with only the protein values shown (other measurements showed similar results). Marked inhibition of growth of both HT-29 and WI-38 cells was induced with little accumulation of protein resulting during the 6-day culture period in the presence of PALA. Growth inhibition induced by PALA was found to persist even after the removal of PALA from the growth medium, which is consistent with the demonstrated inability of PALA-treated cells to resume de novo pyrimidine synthesis subsequent to removal of the inhibiting agent (see Table 2). In the presence of either $1 \times 10^{-4}$ or $1 \times 10^{-3}$ mM PALA, little increase in cell protein occurred after several days in culture, with the existing low levels of protein maintained during the subsequent culture period in the absence of PALA. Cultures treated with the higher PALA concentration contained only slightly less cell protein as shown.

**Metabolic Effects of Pyrimidine Deprivation.** The effect of PALA-induced inhibition of pyrimidine synthesis on cell...
viability was examined on the basis of glucose utilization rates. In Chart 3 are shown the relative rates of glucose utilization by PALA-treated and control HT-29 and WI-38 cells. Little impairment of glucose utilization was evident in either of the cells as a consequence of inhibition of pyrimidine (and purine) synthesis by PALA as shown. The HT-29 neoplastic cells were found to exhibit a higher rate of glucose utilization than the normal diploid WI-38 cells.

The metabolic effects of PALA-induced pyrimidine deprivation were examined further in relation to glycogen synthesis. Participation of uridine nucleotides in glycogen synthesis involves UDP as carrier and UDP-glucose as the ultimate glucosyl donor to the amylose chain. Since the normal WI-38 cell and the neoplastic HT-29 cell were found to engage in an active glycogen synthesis, both were tested to determine the resultant effect of PALA-induced pyrimidine deprivation on their ability to synthesize glycogen. In Chart 4 are shown relative rates of glycogen restoration in PALA-treated and control cells that had previously been maintained in the absence of glucose to reduce existing glycogen stores. In contrast to the control cells, little accumulation of glycogen resulted on incubation of the PALA-treated WI-38 and HT-29 cells in the presence of excess glucose. Impairment of glycogen metabolism as a consequence of cellular pyrimidine deficiency was evident from these studies. Glycogen synthesis in the control cells was found to be more rapid in the WI-38 as compared to the HT-29 cells.

DNA Synthesis in the Pyrimidine-deprived Cells. PALA-treated cells were examined in relation to DNA synthesis. During and subsequent to PALA treatment, little net accumulation of cell protein was found to occur (see Chart 2), indicative of complete growth inhibition. On the other hand, the possibility that some proliferative growth was occurring which was balanced by a PALA-induced, enhanced rate of cellular destruction (due to synthesis of defective cells?) could not be excluded. To examine this possibility the DNA of HT-29 and WI-38 cells was labeled with [3H]-thymidine, and the cells were subsequently cultured in the presence and absence of PALA. Dilution of the label (decrease in specific activity) was followed relative to the DNA content of the cells during the subsequent culture periods. The results of a representative experiment obtained with HT-29 cells are shown in Chart 5. Generally similar findings were obtained with WI-38 cells and are not shown. During the 11-day period examined, the control cultures increased their DNA content 7-fold, whereas the PALA-treated cultures showed little accumulation of DNA. The specific activity (cpm/μg) of the DNA in the control cultures decreased about 7-fold (from a high of 37 to 5), correlating well with the measured accumulation of DNA. The specific activity of the DNA in PALA-treated cells decreased 4.5-fold (from a high of 34 to 7.5), with little associated accumulation of DNA occurring. These results would imply that considerable
DNA synthesis, i.e., proliferative growth, was occurring in the PALA-treated cells (60% of the control rate), balanced by equivalent cellular destruction. On this basis, the PALA-treated cells would appear to represent a steady-state population in which new cells are being continually formed under conditions of pyrimidine deprivation, leading to a reduced survival ability. New cell production would be supported by a limited source of pyrimidine, available through salvage mechanisms from the dying cells with the balance obtained by leakage through the inhibited de novo pathway.

Growth Inhibition by PALA, Prevention and Reversal by Uridine. Growth inhibition of HT-29 cells by PALA could be prevented by supplying the cells with the preformed pyrimidines, cytidine and uridine, in confirmation of selective inhibition of de novo pyrimidine synthesis by PALA as the basis of its antiproliferative action. Cells cultured for 6 days in the presence of both PALA and uridine (each at 0.1 mM) and then for 6 days in their absence showed little impaired growth compared to control cultures as illustrated in Chart 6 (compare Curves 1 and 2). On reducing uridine concentrations, growth inhibition by PALA was increasingly evident (not shown). Cells cultured with PALA alone for 6 days and then for 6 days in its absence, showed little growth (see Chart 6, Curve 4). The sustained growth inhibition demonstrated by the PALA-treated cells even after removal of the inhibitor from the culture medium was examined further. In this regard, it was shown previously (Table 2) that PALA-treated cells did not resume de novo pyrimidine synthesis on subsequent culture in the absence of the inhibitor. Inhibition of pyrimidine synthesis by PALA, as a competitive inhibitor of aspartate transcarbamylase (see Chart 1), would be expected to be freely reversible on removal of PALA from the culture medium. The inability to restore pyrimidine synthesis after PALA removal was considered, therefore, to represent a further secondary impairment of the pathway resulting as a consequence of PALA action. Whether the continued impairment of pyrimidine synthesis was the sole cause of the inability of the PALA-treated cells to resume growth was tested. In this case, cells cultured initially in the presence of PALA were supplied with pyrimidine (uridine) subsequent to the removal of PALA (see Chart 6, Curves 3a, b, and c). The sustained growth inhibition induced by PALA was found to be reversed by providing the cells with preformed pyrimidine. Uridine supplied at 0.1 mM restored growth to nearly control rates (compare slopes in Chart 6, Curves 1 and 3a) in the PALA-treated cells. It was concluded from these findings that pyrimidine deprivation was the sole cause of the growth inhibition of HT-29 cells observed both during and after PALA treatment.

Growth Inhibition by PALA and Dose Schedules. Effective PALA dose schedules, with respect to duration and frequency, which resulted in complete inhibition of growth of HT-29 cells were as follows. Cells maintained in the presence of 1 mM PALA for at least 2 consecutive days showed little growth thereafter within a 26-day culture period examined. Single 24-hr dose schedules repeated at intervals of 3, 5, 7, and 9 days were also effective in preventing growth. The short-term effects of PALA showed little distinguishable gross morphological effects on cell populations; however, with prolonged culture, the cells appeared to flatten and spread and contained increasing numbers of vacuoles (both HT-29 and WI-38). Of particular interest was the appearance of small colonies of apparent mutant cells in the growth-inhibited cultures which were maintained for prolonged periods. Selection of these mutant strains and their further study are in progress.

Effect of Pyrimidine Analogs on Growth of HT-29 Cells. Comparative growth inhibition patterns of HT-29 cells cultured in the presence of selected pyrimidine analogs are shown in Chart 7. Little growth inhibition was induced by 6-azauridine when tested at concentrations up to 0.1 mM. Cells cultured in the presence of cytosine arabinoside and 5-fluorouracil failed to grow at minimal concentrations of each at 1 and 10 μM, respectively. Complete growth inhibition induced in the presence of 1 μM cytosine arabinoside was not sustained on removal of the inhibiting agent from
the culture medium (Chart 7, Curve 3). On the other hand, growth inhibition induced by 5-fluorouracil at 10 μM continued even after its removal from the culture medium, similar to the inhibition pattern obtained with PALA (Chart 7; compare Curves 4 and 5).

Antitumor Activity of PALA in Mice with Colonic Tumor Transplants. The antitumor activity of PALA was tested in vivo. In these studies, mice bearing a transplantable colonic tumor were treated with PALA to determine its effect as an antitumor agent. The results of 3 preliminary experiments are summarized in Table 3. The tumor used in these studies was highly metastatic and resulted in relatively short mean survival times of the host animals as shown by the 3 sets of controls. In Experiment 3, a longer mean survival time of the control group of animals was found relative to the initial 2 experiments, presumably due to the use of older and larger animals. Within each experiment, however, the administration of PALA resulted in both delay in growth of the tumor transplant and extension of the mean survival time of the host animals relative to the corresponding controls. PALA was administered in these experiments at concentrations ranging from 230 to 360 mg/kg body weight per i.p. injection, with the administration repeated 3 to 4 times at the intervals indicated. Extension of mean survival time by PALA ranged from 47 to 56% in the 3 experiments conducted. Simultaneous administration of cytosine arabinoside or 5-fluorouracil in combination with PALA was also examined in a preliminary manner. Although the combinations did not result in any remarkable extensions of mean survival times beyond that obtained with PALA alone, further combination studies with cytosine arabinoside were indicated (mean survival time extended 75% in 1 experiment). The concentrations of PALA administered in these studies were below the toxicity levels by a factor of 2 to 3 times. Alternative routes of PALA administration, optimal treatment schedules, and combination chemotherapy have yet to be examined systematically. Whether simultaneous administration of uridine with PALA would reverse the antitumor activity of PALA in vivo was not tested.

DISCUSSION

De novo pyrimidine synthesis was determined in these studies by measuring 14C02 incorporation into uracil, cytosine, and thymine prepared in high radiochemical purity from all cell sources (i.e., nucleotides and nucleic acids) treated as a common pool. The method utilized improves on

Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Effect of PALA alone and in combination with cytosine arabinoside or 5-fluorouracil on survival times of BALB/c mice bearing line 26 colonic tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice</td>
</tr>
<tr>
<td>Experiment 1*</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>PALA</td>
</tr>
<tr>
<td></td>
<td>PALA + cytosine arabinoside</td>
</tr>
<tr>
<td>Experiment 2*</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>PALA</td>
</tr>
<tr>
<td></td>
<td>PALA + cytosine arabinoside</td>
</tr>
<tr>
<td></td>
<td>PALA + 5-fluorouracil</td>
</tr>
<tr>
<td>Experiment 3*</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>PALA</td>
</tr>
</tbody>
</table>

* PALA was given i.p. either alone at 360 mg/kg or combined with cytosine arabinoside at 17.4 mg/kg or of Days 1, 4, and 7, after tumor cell implants.

* Significance value on Student's t test. Mean survival times relative to controls.

* PALA was given i.p. alone at 230 mg/kg or at 343 mg/kg with cytosine arabinoside at 24 mg/kg or with 5-fluorouracil at 13 mg/kg on Days 1, 4, 7, and 14, after tumor cell implants.

* PALA was given i.p. at 343 mg/kg on Days 1, 4, 7, and 10, after tumor cell implants.
that reported by others (e.g., see Ref. 8) in which pyrimidine synthesis was estimated by measuring $^{14}$CO$_2$ incorporation only into the acid-soluble uridine nucleotides, which represents a transient pool of varying size and turnover. The method also offers the advantage of allowing simultaneous determination of de novo purine synthesis, by measuring $^{14}$CO$_2$ incorporation into adenine and guanine which are also isolated (see Fig. 1).

Pyrimidine synthesis was found to be blocked in cells treated with PALA, which acts as a competitive inhibitor of aspartate transcarbamylase. Pyrimidine synthesis was not restored on removal of PALA, indicating either a secondary derangement of the de novo pathway or an unexplained prolonged retention of PALA by the cells.

The PALA-treated, pyrimidine-deprived cells could be maintained as viable cultures for prolonged periods without net change in cell numbers. Such cultures of HT-29 cells eventually developed mutant cell colonies (also found with other cell types [10]). Metabolic studies showed the PALA-treated cells to be capable of utilizing glucose at normal rates; however, some impairment was found in their ability to synthesize glycogen, indicative of a deficiency of uridine nucleotide.

The PALA-treated, HT-29 malignant cells, although appearing to have shifted from a normal proliferative state to a quiescent state, continued to synthesize DNA. A steady-state cell population was thus indicated in which new cells were being produced, of limited survival ability due to pyrimidine deprivation, in equal numbers to cell deaths. This would be consistent with the proposal by Pardee (13) that malignant cells demonstrate limited survival ability under adverse conditions (e.g., pyrimidine deficiency) due to loss of restriction point control and stop randomly in their division cycle and die.

That pyrimidine deprivation accounted solely for the sustained growth defect of PALA-treated HT-29 cells was confirmed. By providing an exogenous pyrimidine source (uridine), rapid restoration of normal growth patterns was observed with the PALA-treated cells (Chart 6). The utility of PALA as a highly selective inhibiting agent of cell pyrimidine synthesis was also confirmed by these studies.

Antitumor activity of PALA was tested in in vivo studies against a transplantable colonic tumor in mice. Formulation of drug schedules was aided by some guidelines provided by the in vitro cell culture studies. Our preliminary testing indicated both delay in tumor growth and extension of mean survival time of the host animal by PALA, warranting further study of its antitumor action.

ACKNOWLEDGMENTS

The authors wish to acknowledge valuable consultations with Dr. G. Stark, Dr. T. Yoshida, and Dr. N. Kretchmer, and the excellent technical assistance of M. Boswell and E. Chow.

REFERENCES

Inhibition of Pyrimidine Biosynthesis

Fig. 1. Radioautograph of "C-labeled cell pyrimidines (and purines), after chromatographic resolution to high radiochemical purity. Each of the free bases was first isolated from perchloric acid cell digests by chromatography in isopropyl alcohol/HCl/H2O (65/17/18), purified by adsorption and elution from charcoal, and chromatographed further in water-saturated butyl alcohol (see "Materials and Methods"). The final chromatographed products are shown in the radioautograph. G, guanine; A, adenine; C, cytosine; U, uracil; T, thymine.
Selective Inhibition of Pyrimidine Biosynthesis and Effect on Proliferative Growth of Colonic Cancer Cells

Kenneth K. Tsuboi, Henry N. Edmunds and Linda K. Kwong


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/37/9/3080

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.