Pyrazofurin Enhancement of 5-Azacytidine Antitumor Activity in L5178Y and Human Leukemia Cells

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ABSTRACT

Pyrazofurin (PF), which is an inhibitor of orotidylate decarboxylase (the conversion of orotidine 5'-monophosphate to uridine 5'-monophosphate) results in marked reductions of intracellular levels of uridine triphosphate and cytidine 5'-triphosphate. These triphosphate ribonucleotides are known inhibitors of uridine-cytidine kinase. Exogenous uridine is rapidly phosphorylated to uridine 5'-monophosphate by this enzyme in the presence of PF and thereby circumvents the lethal effect of PF. Therefore, the effect of PF treatment on the cellular metabolism of 5-azacytidine (5-aza-C), a nucleoside drug analog of cytidine, was studied in the experimental tumor cell L5178Y and human leukemia cells.

PF, in concentrations (5 x 10^{-4} M) which inhibited orotidylate decarboxylase and produced 80% reduction in uridine triphosphate resulted in more rapid accumulation of 5-aza-C into, and enhanced killing of, rapidly dividing leukemia cells. Ribonucleotide analysis by high-pressure liquid chromatography demonstrated a 400% increase of 5-aza-cytidine 5'-triphosphate in PF-treated cells. This triphosphorylated form of 5-aza-C was incorporated in greater quantities into RNA that resulted in a 40% reduction of [3H]leucine incorporation into protein, indicating the synergistic lethal effects observed with this drug sequence of PF → 5-aza-C were by augmented inhibition of protein synthesis, the previously proposed major antitumor mechanism of 5-aza-C. PF followed by 5-aza-C for the treatment of rapidly proliferating human leukemia may be a useful sequential drug combination when these patients have failed more standard forms of antileukemic therapy.

INTRODUCTION

PF (4-hydroxy-5,8-d-ribofuranosylpyrazole-3-carboximide) is a nucleoside analogue which is phosphorylated to its active form, PF-PO4, by adenosine kinase (EC 2.7.1.20) (4). This monophosphorylated form of PF, which is formed in both L5178Y murine and human leukemia cells, is a competitive inhibitor of orotidylate decarboxylase (EC 4.1.1.23) (K_i ~ 10^{-4} M) (4, 5). The consequence of this de novo pyrimidine synthesis blockade is a marked reduction in the uridine and cytidine ribonucleotide pools (6). In the presence of PF [14C]uridine rapidly accumulates intracellularly in cells replenishing these 2 decreased pyrimidine ribonucleotide pools circumventing the lethal blockade of PF allowing normal cell growth to continue (5).

5-aza-C (4-amino-1-β-D-ribofuranosyl-S-triazine-2-(IH)-one), which was synthesized in 1964 by Piskala and Sorm (24), is a ring analog of the pyrimidine nucleoside cytidine differing only by the replacement of the 5 carbon with a nitrogen. This drug is the newest effective chemotherapeutic agent available for the treatment of human acute myelogenous leukemia (17, 22, 31).

Uridine-cytidine kinase (EC 2.7.1.48) is the enzyme responsible for the phosphorylation of uridine and cytidine in the presence of ATP and Mg^{2+} to UMP and CMP. This enzyme is of considerable importance in the salvage pathway for the pyrimidine ribonucleosides because it can utilize exogenous uridine and cytidine to maintain the pyrimidine ribonucleotide pools (7, 18, 23, 25, 29, 30). 5-aza-C, as would be expected, is also phosphorylated by uridine-cytidine kinase to the monophosphate derivative (20). The triphosphorylated form of 5-aza-C (5-aza-CTP), which is subsequently incorporated primarily into RNA, is thought to be the active form of this drug (8, 9, 16, 21). The RNA formed in the presence of 5-aza-C is modified such that protein synthesis is inhibited (20). In addition to this major effect on protein synthesis, the proposed lethal effect, 5-aza-C is minimally incorporated into DNA (21) and also inhibits orotidylate decarboxylase (8, 28), but the antitumor effect is thought not to be the result of these interactions.

Since PF treatment of cells results in a marked reduction of UTP and CTP, both feedback modulators of uridine-cytidine kinase (20), it is a logical consideration to evaluate the subsequent incorporation of 5-aza-C into these depleted ribonucleotide pools and the resultant effect on cellular metabolism.

The following study is to establish valid biochemical reasons for the potential use of the sequential drug combination of PF followed by 5-aza-C in the treatment of human adult leukemia. Preliminary portions of this work have been previously reported (6).

MATERIALS AND METHODS

Drugs. PF was supplied by Eli Lilly and Co., Indianapolis, Ind. 5-aza-C was provided by the National Cancer Institute, Bethesda, Md. 4-[6-3H]5-aza-C (45 mCi/mmol) was supplied by the Stanford Research Institute, Menlo Park, Calif. under the direction of the National Cancer Institute. It was stored as a powder at −5°C; solutions were prepared in 0.9% NaCl solution immediately before each use. [3HCOOH]Orotate (41.25 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. A sterile solution of [3HCOOH]Orotate...
(2.6 x 10^-5 M, 1.4 x 10^6 cpm/ml) was prepared in 0.9% NaCl solution, and pyrogen-tested in rabbit conjuctiva for subsequent use in human subjects.

Dr. E. Ehrenkaufer of the Brookhaven National Laboratory prepared [3H]PF by radiofrequency excitation of tritium gas (14). Exchangeable tritium was removed by evaporation, and the remaining material was chromatographed on Whatman No. 3 paper with isopropyl alcohol:NH4OH:H2O (7:1:2) and ethanol:1 m ammonium acetate (pH 5.5) (7.3). This was followed by separation on a Dowex 1-X8 (formate, 200 to 400 mesh) with ammonium formate elution. This resulted in complete separation of biologically active [3H]PF (16 mCi/mmol) which was stored at 0° and used within 2 weeks of preparation.

Cells. The murine leukemia, L5178Y, was maintained as ascites tumor by weekly i.p. transfer in CS7BL x DBA/2 F1 (hereafter called BD2F1) female mice purchased from Jackson Laboratories, Bar Harbor, Maine, and also as a suspension culture in Fischer's medium with 10% horse serum at 37° in a 5% CO2 atmosphere. Human leukemia cells from adult patients who gave their informed consent were obtained by venapuncture into heparinized tubes. These cells were immediately separated from RBC by centrifuging at 100 x g for 10 min in 3 times the blood volume of a density gradient medium purchased from Flow Laboratories, Rockville, Md. Microscopic examination consistently demonstrated less than 0.5% contamination with RBC. The separated leukemic cells were then resuspended in Fischer's medium to a concentration of 1 x 10^7/ml, evaluated for exclusion of trypan blue as a crude measure of viability and used immediately for biochemical study. Less than 0.1% of cells failed to exclude trypan blue.

Cloning. The biological antitumor effect of each drug and sequential drug exposure was determined by cloning L5178Y cells in soft agar by a modification of a technique previously reported (10). After the appropriate single drug exposure, the cell suspension was centrifuged at 100 x g for 10 min at 37°. The drug-containing medium was discarded, and the cell pellet was resuspended in drug-free Fischer's medium and then centrifuged as before. This procedure was repeated twice to remove any extracellular drug before cloning. In sequential drug studies the second drug was added to the washed cell suspension after a 2-hr exposure to the first drug. At the designated times the identical washing sequence was done before cloning the cells.

Following the appropriate drug exposure and washing, 100 cells were placed into 10-ml culture tubes which contained 2 ml of liquified agar (37°) and 3 ml of drug-free Fischer's medium containing 10% horse serum, capped, and placed at 37° in a 5% CO2 atmosphere. The consistency of this solution does not allow cell settling or random cell motion but does permit continued growth. Cells that remain viable after the drug exposure, as defined by having the continued capability to divide and produce progeny, will form individual cell colonies (clones) that are visible after 10 days of incubation. The effect of prior drug treatment on the cell's ability to develop progeny (clones) can therefore easily be determined visually and be expressed as a percentage of clones formed from untreated cells. This percentage of clones formed is referred to as viability (clones formed drug-treated cells/clones formed untreated cells × 100 = percentage of viability). The cloning efficiency of control cells was 80%. Experiments were done in triplicate 3 times; the variability was less than 5% and all points represent mean values.

Orotic Acid Metabolism. The metabolism of [14COOH]-orotate was studied in log-phase growth suspension cultures of L5178Y (5 x 10^6 cells/ml) before and after exposure to PF. The [14COOH]orotate upon entry into the cell is phosphorylated to [14COOH]OMP and then rapidly decarboxylated to UMP releasing the label as CO2. The rate of CO2 formed is a measure of the inhibition of the orotidylate decarboxylase.

One liter of L5178Y was divided into 0.5-liter portions and PF (5 x 10^-4 M) added to one portion. After thorough mixing, 5 ml of the cell suspension were rapidly placed into 20-ml plastic scintillation vials that were put into a 37° shaker waterbath in triplicate. After 5 min of PF exposure, 0.1 ml of the [14COOH]orotate solution was added to the cell suspensions, and a cap that had a 21-gauge 2 1/2-inch needle with a NaOH-soaked Whatman No. 3 circular filter paper on the intravial end was immediately placed on the vial. Then at designated times 0.2 ml of 60% HClO4 was placed into the cell suspension via the needle to stop all cellular metabolism. At completion of the experiment, the filter paper was removed, dried, placed in glass minicounting vials with 3 ml of OCS (Amersham/Searle, Arlington Heights, Ill.), and the radioactivity CO2 was determined with a Model 3255 Packard Spectro Flowmeter (Downers Grove, Ill.).

The metabolism of [14COOH]orotate in humans before and after i.v. administration of 200 mg/sq m of PF was studied in 3 patients with leukemia after obtaining informed consent. Of pyrogen-free [14COOH]orotate (2.6 ± 10^-5 M, 1.4 x 10^6 cpm/ml), 5 ml were given by i.v. injection. The patient's breath was collected for 1-min periods in disposable plastic bags at 10-min intervals. After completion of the collection at the bedside, the secured bags were taken to the laboratory where the captured breath was slowly bubbled through a saturated solution of barium hydroxide in a gas washing bottle. The precipitated barium carbonate (BaCO3) was collected, washed 3 times with acetone and water, and dried. Then 100 mg of this precipitate were suspended in 10 ml of scintillation fluid composed of toluene (21%), ethanol (11%), PPO (8 g), POPOP (0.1 cpm), Cab-O-sil (90 g), and counted in a liquid scintillation spectrometer as before.

Nucleotide Metabolism. BD2F1 mice 6 days following an i.p. inoculation of 1 x 10^4 L5178Y cells were given 100 mg/kg of PF i.p., a dose which we previously established as totally inhibitory of orotidylate decarboxylase (EC 4.1.1.23) (4, 5). At designated times following PF treatment, 0.1 ml of [14C]-aza-C (made immediately before use from the solid form; generally 1 x 10^6 cpm/ml) was given i.p. One hour later the mice were killed by cervical traction, the ascites tumor was immediately removed and centrifuged at 1000 x g for 10 min, the supernatant was discarded, and the cell pellet precipitated in 2 ml of 10% TCA. This supernatant, which contains the ribonucleotides, was extracted 3 times with 6 ml of ether to remove the TCA and stored at -20° until all samples could be evaluated on the same day. The
ribonucleotide concentrations in these extracts were determined by high-pressure liquid chromatography on a DuPont (Model 640) high-pressure liquid chromatography system (DuPont Instruments, Wilmington, Del.) with a linear gradient (0.01 to 1.0 m) of sodium phosphate buffer (pH 3.51) with a flow rate of 0.9 ml/min on a Partisil SAX (10-μm particle size) column (25 cm × 4.6 mm). Absorbance was recorded at 254 and 280 nm, and 30-sec fraction collections were obtained for radiochromatography correlation with the chromatograms.

5-aza-C Intracellular Uptake and Incorporation in DNA and RNA. L5178Y cells in log phase growth (5 × 10⁶ cells/ml) were exposed to PF (5 × 10⁻⁶ M). After 1, 3, and 6 hr, 250 ml of the cells were centrifuged at 100 × g × 5 min, the supernatant was discarded, and the cell pellet was resuspended in 5 ml of Fischer’s medium that contained 10% horse serum and 10% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4; 1.25 m) to obtain a cell concentration of 5 × 10⁷ cells/ml, placed in a 25-ml stoppered flask, and placed in a 37° shaker water bath. Then 0.1 ml of [³²P]5-aza-C (1 × 10⁶ cpm/0.1 ml) was added and 1-ml of the cell suspension centrifuged at 100 × g × 5 min in a Weintrobe Sedimentation tube and the ratio of intracellular to extracellular drug determined as previously described by Handschumacher and Fischer (13). The identical procedure was performed with [³H]PF (0.1 ml of 3 × 10⁻⁶ M; 6 × 10⁴ cpm/ml) to determine PF accumulation into human leukaemia cells after separation as previously described.

Following exposure of L5178Y cells to PF as previously, the same concentration of [³²P]5-aza-C was added. After 1 hr, the cells were centrifuged as before, and the pellet was precipitated in 2 ml of 10% TCA. The precipitate was washed with 5 ml of cold 10% TCA until no radioactivity could be detected in 1 ml of the supernatant (7 times). The DNA and RNA were then separated by the method outlined by Trakatellis and Axelrod (27). The radioactivity in the DNA and RNA was determined and related to micrograms of RNA as determined by the orcinol reaction (2).

Protein Synthesis. The protein synthesis of L5178Y cells grown in suspension culture was established by [³H]leucine incorporation into the 10% TCA-precipitated cell pellet. After 1 hr of exposure to 5-aza-C (5 × 10⁻⁶ M) ± PF (5 × 10⁻⁶ M) pretreatment for 1, 3, and 6 hr, 5 μCi of [³H]leucine (60 Ci/mmol) were added for 1 hr, and the cells were precipitated as before and washed with 5 ml of 0.9% NaCl solution until no radioactivity was detectable in 1 ml of supernatant (7 washes). The pellet was then dissolved in 0.5 ml of 1 N NaOH at 37° for 12 hr, and the radioactivity was determined and expressed as cpm/10⁶ cells.

RESULTS

L5178Y. PF at 5 × 10⁻⁶ M completely prevented decarboxylation of [¹⁴C]orotate in L5178Y cells (Chart 1), which is consistent with our previous observation that this dose of PF prevented growth of these cells when present in the suspension culture (5).

The cloning system was used to evaluate the lethal effects of PF and 5-aza-C during short-term drug exposure. PF at 5 × 10⁻⁶ M resulted in no decrease in cell viability at 4 hr, but following a 24-hr exposure only 10.5% of the cells retained their ability to form progeny (clones). 5-aza-C at 5 × 10⁻⁶ M produced only a slightly increased lethal effect at 4 and 24 hr, 55 and 4% viability, respectively. When 5-aza-C preceded the addition of PF by 2 hr, there was no appreciable difference from 5-aza-C alone. However, following 2 hr of PF (5 × 10⁻⁶ M), there were no viable cells when 5-aza-C was given for the next 8 to 24 hr indicating a pronounced sequential synergism (Chart 2).

Since 5-aza-C is a nucleoside analog of cytidine and is phosphorylated by uridine-cytidine kinase, which phosphorylates both uridine and cytidine, and having previously established that the feedback inhibitors of this enzyme (UTP and CTP) (1, 20) are decreased in response to PF, then an evaluation of the incorporation of 5-aza-C into L5178Y is appropriate. As can be seen in Chart 3, PF (5 × 10⁻⁶ M) did result in an enhanced incorporation of 5-aza-C into both the acid-soluble and insoluble fractions.

If this enhanced incorporation of 5-aza-C into cells following exposure to PF is the actual cause of the sequential drug synergy observed, then there should be more 5-aza-CTP, the lethal form of 5-aza-C, present in the ribonucleotide pools. The changes in the [¹⁴C]5-aza-CTP chromatograms following PF are shown in Chart 4. The standards, CTP, UTP, ATP, and GTP each at 2.25 nmol, are in the top panel, the ribonucleotide pool profiles of untreated L5178Y cells are in the second panel. When PF was present for 1 hr, there was a reduction of 80% in UTP (third panel). 5-aza-C alone resulted in a new peak in the triphosphate region located between UTP and ATP, presumably 5-aza-CTP. This new peak increased approximately 400% when PF preceded 5-aza-C by 1 hr (fifth panel). The radiochromatogram demonstrated that all of the radiolabel in the triphosphate region was present in this new peak confirming that this new triphosphate was 5-aza-C. Therefore, the pretreatment with PF did result in more intracellular quantities of the lethal form of 5-aza-C.

However, if this augmented accumulation of 5-aza-CTP is actually a cause of the synergism, then the synthesis of...
Chart 2. Effect of PF and 5-aza-C on the cloning viability of L5178Y. PF (5 x 10^{-6} M) when given alone to the cell suspension before cloning resulted in 10.5% viability after 24 hr, (O). 5-aza-C (5 x 10^{-4} M) resulted in a 4% viability after a 24-hr exposure, (B). There was no deviation from this viability curve when 5-aza-C (5 x 10^{-4} M) preceded the addition of PF (5 x 10^{-4} M) by 2 hr, (O). But when PF (5 x 10^{-4} M) was added to the cell suspension 2 hr before 5-aza-C (5 x 10^{-4} M), no clones developed after the subsequent 8-hr exposure of 5-aza-C (B).

Chart 3. Augmentation of [^{14}C]5-aza-C accumulation in the acid-soluble and insoluble fraction of L5178Y cells. Rate of entry into control (O); after PF (5 x 10^{-4} M) exposure of 1 hr (C); and 6 hr (B). PF resulted in augmentation of [^{14}C]5-aza-C entry into these cellular compartments.

Chart 4. Triphosphate ribonucleotide pool alterations of L5178Y cells and the enhanced accumulation of 5-aza-CTP following PF exposure. The nucleotide pools were evaluated of L5178Y ascites cells extracted from BD2F, mice inoculated 6 days previously with 1 x 10^{6} cells i.p. Top panel, standards CTP, UTP, ATP, and GTP at 2.25 nmol. Second panel, untreated L5178Y cells (control). Third panel, PF (100 mg/kg) given 1 hr before cell harvest. This treatment of PF has resulted in a marked reduction of UTP. Fourth panel, 5-aza-C (100 mg/kg) given i.p. 1 hr before cell harvest results in a new nucleotide presence in the triphosphate region, presumably 5-aza-CTP. Fifth panel, when PF, 100 mg/kg i.p., precedes 5-aza-C (100 mg/kg i.p.) by 1 hr, this new ribonucleotide is increased approximately 400%. Panel 6, radiochromatogram with [^{35}S]-aza-C confirms this new peak to be 5-aza-C, and therefore most likely 5-aza-CTP. (See “Materials and Methods” for details.)

protein, the proposed major lethal effect of 5-aza-CTP, should be further depressed when PF precedes 5-aza-C. Chart 5 demonstrates that, following a 6-hr exposure of PF (5 x 10^{-6} M), there was only a minimal effect on incorporation of [^{3}H]leucine into protein of L5178Y cells. 5-aza-C (1 x 10^{-6} M) exposure for 1 hr did not alter the incorporation of [^{3}H]leucine either. But when PF (5 x 10^{-6} M) preceded a 1-hr exposure of 5-aza-C (1 x 10^{-6} M) by 6 hr, there was a 40% decrease in [^{3}H]leucine incorporation into protein. This suggests that the increased 5-aza-CTP was having an enhanced effect on protein synthesis and was possibly one explanation for the sequential effects observed in the cloning system.

Since the effect of 5-aza-CTP on protein synthesis would probably be mediated from the incorporation of this triphosphate into RNA, this was examined. As the hr of PF exposure were increased before adding [^{14}C]5-aza-C for 1 hr, there was an augmented incorporation of labeled 5-aza-C into RNA which was maximum at 3 hr (Chart 6). Of the total amount of label present in nucleic acid, 78% was in the RNA fraction of the non-PF exposed cells (time 0). This
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Chart 6. Incorporation of \(^{14}C\)-5-aza-C into RNA and DNA of L5178Y suspension cells following PF, \(5 \times 10^{-6}\) M. The maximum incorporation of a 1-hr pulse of labeled 5-aza-C in RNA was after a 3-hr exposure of PF. The reduction in DNA may be a reflection of inhibition of DNA synthesis in some cells exposed to this drug sequence.

is similar to what has been noted previously when cells were exposed to 5-aza-C alone (21). However, when PF preceded 5-aza-C the percentage of the total amount of 5-aza-C entering the RNA fraction increased to 90%. The decrease of labeled 5-aza-C incorporated into DNA may partly be a reflection of depressed DNA synthesis in cells that are unable to undergo division.

Human Studies. Orotic acid metabolism was studied in 3 leukemic patients. When the \(^{14}COOH\)orotate was administered i.v. without prior PF treatment, 99% of the label was recovered as exhaled \(^{14}CO_2\). Only a minimal quantity of \(^{14}CO_2\) was released into the breath when the \(^{14}COOH\)orotate was given 5 min following the i.v. administration of 200 mg/ sq m of PF. This marked inhibition of decarboxylation of orotate persisted for at least 24 hr and was only 75% of control values 1 week after the single PF dose (Chart 7). Urine was also collected, and the radioactivity was determined. During the time of PF inhibition of decarboxylation, 99% of the injected labeled orotate was present in the urine as orotate and orotidine with approximately 70% of the total being excreted during the first 8 hr and the remainder in the next 24 hr (data not shown). On any given day following the PF injection, when orotate metabolism was studied, the sum of the radioactivity present in both the breath and urine was 99% of that which was injected. These findings indicated a pronounced effect of PF on the ability of the human body to decarboxylate orotate. The majority of orotate metabolism in humans is probably in the liver; therefore, these results cannot be interpreted to be representative of the inhibition of orotidylate decarboxylase occurring in leukemia cells. Also, since large increases of orotate occur following PF, some of the apparent decrease in decarboxylation of labeled orotate may be a dilutional effect.

If the sequence of PF followed by 5-aza-C has potential for being synergistic in killing leukemia cells, then based on the previous data presented, the PF must inhibit de novo pyrimidine synthesis of these neoplastic cells.

The entry of \(^{3}H\)PF into leukemia cells from a patient with acute myelogenous leukemia and a patient with chronic myelogenous leukemia was evaluated. PF accumulated in both of these leukemia cell populations. Chart 8 shows only the chronic myelogenous leukemia cells, but it is representative of the other patient studied. As previously reported (5), PF monophosphate, the active form of the drug, was formed.

Leukemia cells from a patient with acute myelogenous leukemia whose total peripheral leukemia cell count was doubling daily were removed and prepared for \(^{14}COOH\)orotate metabolism and intracellular accumulation of \(^{14}C\)-5-aza-C studies as described in "Materials and Methods." As seen in Chart 9, PF completely inhibited decarboxylation of the \(^{14}COOH\)orotate. The accumulation of \(^{14}C\)-5-aza-C into these human leukemic cells was enhanced following a 3-hr in vitro exposure to PF, presumably the consequence of the decreasing UTP and CTP pools as observed with the previous L5178Y cells.

One of the 3 patients above whose total body orotate
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sq m), leukemic cells were removed, and the intracellular accumulation of \[^{14}\text{C}]\text{5-aza-C}\) was examined. There was a 3-fold increase in intracellular labeled 5-aza-C 12 hr after the PF (Chart 10). This indicates that pretreatment with PF before administering 5-aza-C may be potentially clinically useful. Two other patients who also had rapidly increasing leukemia cells had a 3-fold augmented intracellular accumulation of 5-aza-C following PF treatment (data not shown). However, in 3 leukemia patients whose neoplastic cells were not increasing, this augmented 5-aza-C accumulation into cells was not observed. This perhaps indicates that in these later situations the cells were not actively involved in DNA and or RNA synthesis and, therefore, that the UTP and CTP pools were not being depleted in the

Chart 7. \[^{14}\text{COOH}\]orotate metabolism in 3 patients with leukemia. (See "Materials and Methods" for procedure.) The evolution of \[^{14}\text{CO}_2\] into collected breath samples was measured as \[^{14}\text{CO}_2\]barium and was rapid before the i.v. injection of 200 mg/sq m of PF (•). There was no detectable radioactivity 15 min (×) and 24 hr (□) following the injection of PF. There was minimal amount of radioactivity present in the breath 5 days following the PF injection (Δ). Only 75% of the radioactivity was found in the breath after the 7th day of PF treatment (○). During periods of inhibition of decarboxylation of \[^{14}\text{COOH}\]orotate, the radioactivity was present in the urine as orotate and orotidine; the recovery of the total amount of radioactivity in breath and urine was always 99% of that injected. \[^{14}\text{COOH}\]orotate → OMP

--→ UMP

\[^{14}\text{CO}_2\]

Chart 8. Intracellular accumulation of \[^{3}\text{H}]\text{PF}\) into human chronic myelogenous leukemia cells (see "Materials and Methods"). Active accumulation of PF was near maximum at 60 min with the amount of PF inside the cell being 14 times greater than that which was present in the medium.

metabolism was studied also had acute myelogenous leukemia with daily doubling leukemia cells in the peripheral blood. At 1 and 12 hr following an i.v. dose of PF (200 mg/
presence of inhibition of de novo pyrimidine synthesis by PF. Neither entry of PF into nor orotate metabolism of these cells was studied, so the problem could also be an innate resistance of these cells to PF.

**DISCUSSION**

PF in the monophosphate form has a pronounced inhibitory effect on orotidylate decarboxylase as demonstrated by virtually no decarboxylation of [14COOH]orotate in both L5178Y murine and human leukemia cells. As a consequence of this block in the de novo pyrimidine synthesis pathway, there is a marked reduction in the pyrimidine ribonucleotides UTP and CTP and an increase in orotate and orotidine. Uridine, which is phosphorylated by uridine-cytidine kinase, can rapidly enter cells circumventing the otherwise lethal effects of PF. 5-aza-C, a nucleoside drug analog of cytidine, is also phosphorylated by this enzyme and accumulates at an increased rate in L5178Y and human leukemic cells treated with PF. This is presumably the consequence of the decrease in UTP and CTP, the natural feedback inhibitors of uridine-cytidine kinase. This enhanced phosphorylation of 5-aza-C results in increased quantities of 5-aza-CTP, the active metabolite of this drug which subsequently accumulates in RNA and probably accounts for the observed decrease in protein synthesis, the presumed lethal mechanism of 5-aza-C. The proposed mechanism of this sequential synergism is presented in Chart 11.

This biochemical study could explain why the sequence of PF followed by 5-aza-C results in synergistic killing of L5178Y cells in the soft agar cloning system. Since the same biochemical effects also occur in rapidly proliferating human leukemic cells, then it would be logical to use this sequential drug combination in selected leukemia patients when other standard forms of therapy have failed. Because the augmented phosphorylation of 5-aza-C is dependent on depletion of the pyrimidine ribonucleotides, this synergism would be expected to occur only in patients whose leukemia cells are rapidly undergoing DNA and/or RNA synthesis. In vitro evaluation of the orotate metabolism and 5-aza-C accumulation into human leukemia cells in the presence of PF may be a useful predictor of the efficacy of this drug sequence. Obviously normal cells, which are undergoing rapid nucleic acid synthesis, may also be affected by this sequence; for example, normal bone marrow elements and gastrointestinal epithelium. There could, therefore, also be enhanced toxicity with these drugs when given in the sequence described.

Because of the poor sustained remission and survival rates of human adult leukemia with the use of our current drug programs (11, 12, 15, 32, 33), multiple drug therapy based on biochemical and biological drug interactions may be an important aspect to consider in attempting to design future therapy of this disease.

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**REFERENCES**


**Figure 11**

**Figure 11.** Effect of PF block in pyrimidine synthesis and the subsequent enhancement of 5-aza-C accumulation in cells. PF, which is phosphorylated by adenine kinase (1) to PF monophosphate, inhibits orotidylate decarboxylase (2), and as a consequence, the uridine and cytidine ribonucleotides are decreased. The cell, in an attempt to maintain these nucleotide pools, will increase its uptake of exogenous uridine via uridine-cytidine kinase (3). If, however, the PF-treated cells are exposed to 5-aza-C, an analog of cytidine, it will readily be phosphorylated by the now uninhibited uridine-cytidine kinase (3) which can be observed as augmented 5-aza-C accumulation into these cells. The result is enhanced cell killing.
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