Biochemical Pharmacology, Vol. 39, No. 3, pp. 455–462, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

INHIBITION OF PYRIMIDINE METABOLISM IN MYELOID LEUKEMIA CELLS BY TRIAZOLE AND PYRAZOLE NUCLEOSIDES

STEVEN S. MATSUMOTO,* JAMES M. FUJITAKI, L. DEE NORD, RANDALL C. WILLIS, VINCENT M. LEE, BRAHMA S. SHARMA, YOGESH S. SANGHVI, GANESH D. KINI, GANAPATHI R. REVANKAR, ROLAND K. ROBINS, WELDON B. JOLLEY and ROBERTS A. SMITH

Nucleic Acid Research Institute, Costa Mesa, CA 92626, U.S.A.

(*Received* 13 *February* 1989; *accepted* 5 *July* 1989)

Abstract—Two triazole nucleosides, **1** (3-β-D-ribofuranosyl-1,2,4-triazole-5-carboxamide) and **2** (2-β-D-ribofuranosyl-1,2,3-triazole-4,5-dicarboxamide), and a pyrazole nucleoside, **3** (1-β-D-ribofuranosylpyrazole-3,4-dicarboxamide), were found to inhibit pyrimidine nucleotide biosynthesis in the human myeloid leukemia cell line, K562. Cells treated with these inhibitors released orotate in quantities of 8–35 nmol/10^5 cells/day. Treatment with these compounds caused the K562 cells to accumulate in the S phase of the cell cycle and induced the cells to synthesize hemoglobin.

Nucleosides containing a five-membered heterocycle with a carboxamide substituent exhibit a wide range of biological activities. The present work shows that certain novel carboxamide-substituted triazole and pyrazole nucleosides, compounds **1**, **2**, and **3** (Fig. 1), inhibit pyrimidine nucleotide biosynthesis. The cellular responses to these inhibitors of pyrimidine metabolism are compared with the responses to other structural analogs (Fig. 2) including ribavirin (**4**) which has broad spectrum antiviral activity [1] and tiazofurin (**5**) which has anti-tumor activity and has been used as a clinical treatment for myeloid leukemia [2]. Ribavirin and tiazofurin inhibit the IMP dehydrogenase catalyzed-reaction in purine nucleotide metabolism [3].

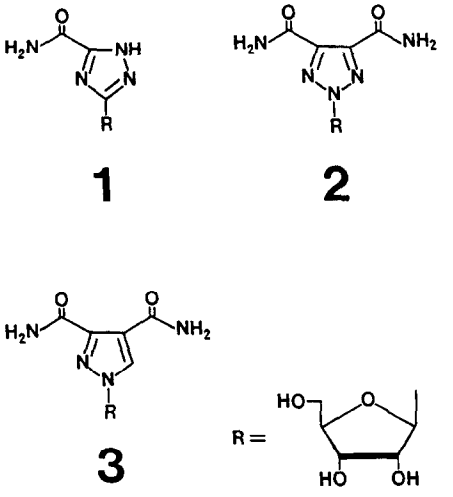

Fig. 1. Structures of novel nucleosides that inhibit pyrimidine biosynthesis. Compound **1**: 3-β-D-ribofuranosyl-1,2,4-triazole-5-carboxamide; compound **2**: 2-β-D-ribofuranosyl-1,2,3-triazole-4,5-dicarboxamide; and compound **3**: 1-β-D-ribofuranosylpyrazole-3,4-dicarboxamide.

MATERIALS AND METHODS

Cell culture. A myeloid cell line, K562, was obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine at 37° in an atmosphere of 5% CO_2 in air. Cell densities were maintained between 0.5×10^5 and 15×10^5 cells per mL. Cell numbers were determined with a Coulter Counter model ZB. Cell viabilities were determined with trypan blue stain. In our hands, the doubling time of the K562 cells was 28 ± 3 hr.

Synthesis of compounds. All of the nucleosides (Figs 1 and 2) were synthesized at the Nucleic Acid Research Institute by published procedures [4–10].

Measurement of nucleosides and nucleotides. High pressure liquid chromatography was used to quantitate the nucleotides and nucleosides in cell and medium fractions following incubation with the compounds. Following incubation of K562 cells in culture medium with the compounds at concentrations indicated in the figures, an aliquot of suspension containing 10^7 cells was centrifuged, and the supernatant medium was separated from the cell pellet. The macromolecules in each fraction were precipitated at 0° by mixing equal volumes of supernatant fraction and 0.8 N $HClO_4$ or by adding 200 μL of ice-cold 0.4 N $HClO_4$ to the cell pellet. The precipitates were removed by centrifugation at 14,900 *g* for 40 sec, and the acid extract was neutralized with trioctylamine in Freon [11]. The nucleosides and bases were separated by reverse-phase chromatography on a Whatman Partisil 5 ODS-3 or an Altex Ultrasphere ODS column (4.6 × 250 mm) at a flow rate of 1.0 mL/min with a gradient of solution A (10 mM potassium

* Correspondence: Steven Matsumoto, Ph.D., Allergan, Inc., 2525 Dupont Drive, Irvine, CA 92715.

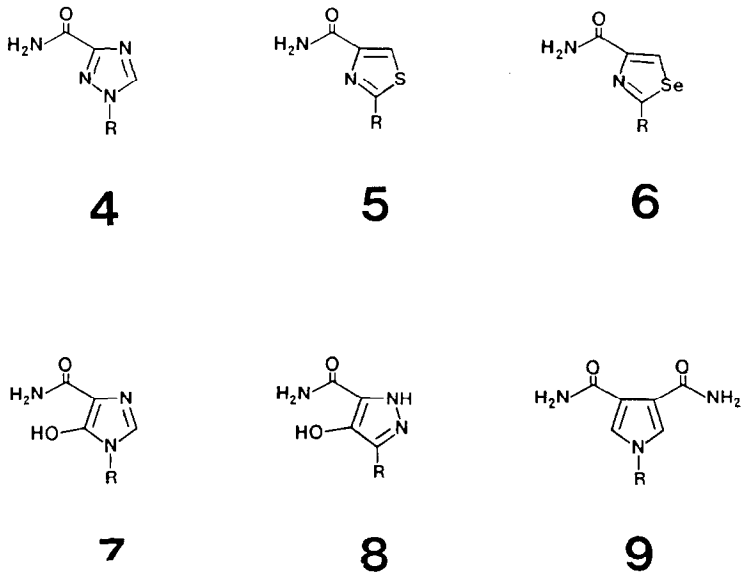

Fig. 2. Structures of related nucleosides. Trivial names: **4**, ribavirin; **5**, tiazofurin; **6**, selenazofurin; **7**, bredinin; **8**, pyrazofurin; and **9**, pyrrole dicarboxamide.

phosphate, pH 3.83) and solution B (60%, v/v, acetonitrile in water) added in the proportions: 0 to 2 min, 0% solution B; 2 to 22 min, 0 to 20% solution B. The nucleotides were separated by anion-exchange chromatography on a Whatman Partisil SAX column (4.6 × 250 mm) at a flow rate of 1.5 mL/min with a gradient of solution A (10 mM potassium phosphate, pH 3.83) and solution C (0.5 M potassium chloride, 0.35 M potassium phosphate, pH 3.6) added in the proportions: 0 to 5 min, 0% solution C; 5 to 11 min, 0 to 30% solution C; 11 to 12.5 min, 30 to 60% solution C; 12.5 to 22.5 min, 60 to 100% solution C. High performance liquid chromatography was performed on a Beckman 110B instrument with a 421A controller or a Hitachi 655A-11 instrument with an L-500 controller or an LKB 2150 instrument with a 2152 controller. Ultraviolet absorbance was detected with an LKB 2140 Rapid Detector or a Kratos Spectroflow 757. Radioactivity was detected with a Ramona Raytest LS. Data acquisition and analysis were performed using Nelson Analytical 2600 Chromatography Software version 3.6 or LKB Wavescan Software version 1.08. The identity of peaks in the column elution profiles was determined by comparison with the retention times and absorbance spectra of known compounds.

Pyrimidine de novo *synthesis.* The incorporation of radioactive bicarbonate into nucleotides was used to measure pyrimidine biosynthesis. K562 cells were incubated with compounds for 3 hr in the cell culture medium at 37°. Then 10^7 cells were resuspended in 10 mL of bicarbonate-free culture medium, 0.1 mCi of [^{14}C]bicarbonate (56 mCi/mmol, ICN Biomedicals, Costa Mesa, CA) was added, and the suspension was incubated for 1 hr at 37°. Extraction of the cell suspension and analysis by anion-exchange chromatography with radioactivity detection was performed as described above.

Purine de novo *synthesis.* The incorporation of radioactive formate into purine nucleosides and nucleotides was measured [12, 13]. K562 cells were incubated with compounds for 20 hr in culture medium at 37°, then 5 μCi of [^{14}C]formate (57 mCi/mmol, ICN Biomedicals) was added to 10^6 cells suspended in 1 mL medium, and incubation was continued for 1 hr at 37°. The cells and medium were separated by centrifugation, and $HClO_4$ was added to both to give a final concentration of 0.4 N. The acidified suspensions were heated in a boiling water bath for 1 hr to hydrolyze nucleic acids and nucleotides to purine bases. The suspensions were centrifuged and the supernatant fractions were applied onto 1-mL Dowex-50 columns. After washing the columns with 10 mL of 0.1 N HCl, the purine bases were eluted off with 5 mL of 6 N HCl, and the radioactivity of a 1-mL aliquot was measured by liquid scintillation counting. Additional 1-mL aliquots were evaporated to dryness, redissolved in 0.1 M $(NH_4)_2PO_4$, pH 3.3, and analyzed on a Whatman Partisil SCX column (4.6 × 250 mm) by elution with 0.1 M $(NH_4)_2PO_4$, pH 3.3, at 1.0 mL/min. Purine bases were detected by ultraviolet absorbance at 254 nm and by radioactivity monitoring.

IMP dehydrogenase assay in whole cells. The activity of IMP dehydrogenase in intact cells was determined by measuring the release of the tritium atom at position 2 from [2,8-^{3}H]inosine [14, 15]. [2,8-^{3}H]Inosine was synthesized from [2,8-^{3}H]adenosine (37 Ci/mmol, ICN Biomedicals) by incubating for 10 min with 2.46 units of adenosine deaminase (Calbiochem, San Diego, CA). To measure IMP dehydrogenase activity, the procedure of Kalman and coworkers [14, 15] was followed. In brief, a suspension containing K562 cells (1.2×10^6 cells/mL) that had been incubated for 20 hr in the absence or presence of compounds was made 5 μM in allopurinol and 1.2 μM in [2,8-^{3}H]inosine (37 Ci/mmol), and incubation was continued for 40 min at 37°. The reaction was stopped by mixing equal volumes of the cell suspension and a 15% acid-treated, activated

charcoal suspension in 4% $HClO_4$. The suspension was centrifuged, and the radioactivity that remained in the supernatant fraction was measured by liquid scintillation counting.

Flow cytometry. Cells (2×10^6) were washed free of growth medium and suspended in 1 mL of an ice-cold, saline solution containing 0.145 M NaCl, 9.5 mM Na_2HPO_4, 2.7 mM KH_2PO_4, 0.1% sodium azide. The cells were fixed by rapidly adding 2 mL of ice-cold methyl alcohol with vigorous mixing. The fixed cells were stored at 4° and were stained on the same day as flow cytometry was performed. The fixed cells were stained by resuspending in 250 μL of the saline solution, incubating with 250 μL of 2 mg/mL ribonuclease A for 30 min at 37°, and incubating with 500 μL of 2 μg/mL propidium iodide in 2.2% sodium citrate for 30 min at room temperature in the dark. The stained cells were protected from light prior to flow cytometric analysis on a Coulter EPICS C cell sorter equipped with a 5-W argon laser. The propidium iodide was excited at a wavelength of 488 nm and the fluorescence was measured with a 590 nm long-pass interference filter.

Measurement of intracellular hemoglobin. The production of hemoglobin by K562 cells was measured by adding 15 μL of a solution containing 0.01% tetramethylbenzidine, 0.15% H_2O_2, and 0.5% acetic acid to an equal volume of the cell suspension and counting the blue-stained cells using a microscope [16]. The results are reported as the percent of the total cells that were positive for the stain, and the low percent of staining of untreated control cells was subtracted in all cases.

RESULTS

Biochemical sites of action. The triazole and pyrazole nucleosides, **1**, **2**, and **3** (Fig. 1), caused K562 cells to accumulate large amounts of orotidine intracellularly and to release large amounts of orotate into the cell culture medium (Figs 3 and 4). The release of orotate shown in Fig. 4 was observed at concentrations of the compounds which were approximately equal to the IC_{50} values for growth inhibition (see below). The release of orotate by cells that were incubated with compounds **1**, **2**, and **3** at lower concentrations (50, 5, and 50 μM respectively) was of nearly the same magnitude as that shown in Fig. 4. Direct evidence that these compounds inhibited pyrimidine *de novo* synthesis was observed in labeling experiments with [^{14}C]bicarbonate. In the presence of compound **1**, **2**, or **3**, the synthesis of [^{14}C]UTP was reduced to 10% or less of the control value, while the total pool of UTP, as measured by ultraviolet absorbance, decreased to 81% of the control value. Furthermore, in the presence of compound **1**, large peaks corresponding to radioactive carbamylaspartate and orotidine appeared (Fig. 5). Similar results were observed with compounds **2** and **3**. Compounds **7** (bredinin) and **8** (pyrazofurin) also caused the cells to release orotate into the medium (Fig. 4). There was no detectable excretion of orotate by cells incubated with compounds **4**–**6**.

Orotate was also produced by a human promyelocytic cell line, HL-60, and a human B-lymphoblast cell line, WI-L2, during incubation with compound

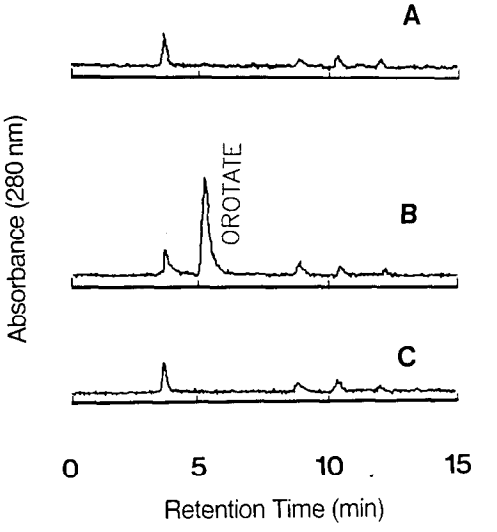

Fig. 3. Nucleosides and bases released into the medium by K562 cells treated with compound **3** or **4** (the numerals refer to the compounds shown in Figs 1 and 2). Medium samples were extracted and analyzed by reverse phase high pressure liquid chromatography as described in Materials and Methods. Key: (A) untreated, control culture; (B) culture treated with 190 μM compound **3** for 5 days; and (C) culture treated with 40 μM compound **4** for 5 days.

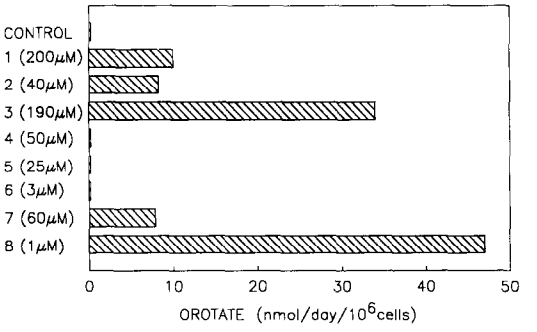

Fig. 4. Orotate released into the medium by K562 cells treated with compounds **1**–**8**. K562 cells were incubated with the compounds (the numerals refer to the compounds shown in Figs 1 and 2) at the concentrations shown in parentheses for 1–5 days. Medium samples were extracted and analyzed by reverse phase high pressure liquid chromatography as described in Materials and Methods.

1, **2**, or **3** (data not shown). However, a mutant of WI-L2 which is deficient in adenosine kinase [17] did not produce orotate during incubation with compound **3**. Furthermore, when the adenosine kinase-deficient mutant was incubated with compound **2** or **3** and [^{14}C]bicarbonate, the accumulation of radioactive carbamylaspartate was greatly reduced. This implies that compounds **2** and **3** must have been phosphorylated by adenosine kinase in order to inhibit pyrimidine biosynthesis. Compound **1** was not tested with the adenosine kinase deficient cell line. Similar evidence for the phosphorylation of compounds **4**, **5**, and **8** by adenosine kinase has been published [17–19].

The specificity of compounds **1**–**3** as inhibitors of pyrimidine biosynthesis was shown by comparison

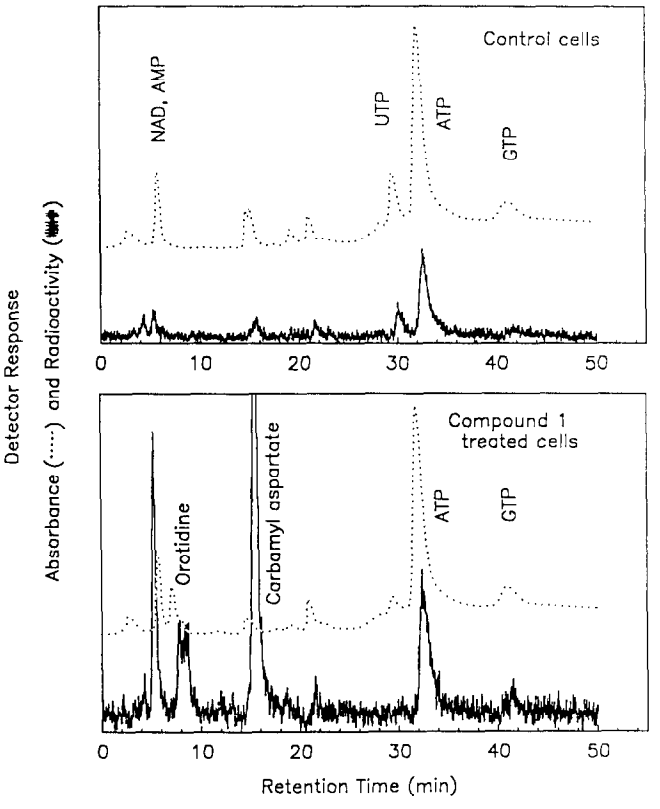

Fig. 5. Effect of compound **1** on the incorporation of [^{14}C]bicarbonate into cellular nucleotides. K562 cells were incubated in medium alone (top panel) or with 20 μM compound **1** for 3 hr (bottom panel), then 0.1 mCi of [^{14}C]bicarbonate was added, and incubation was continued for 1 hr. Cells were extracted and analyzed by anion-exchange high pressure liquid chromatography as described in Materials and Methods. Ultraviolet absorbance at 254 nm (dotted lines); radioactivity (solid lines).

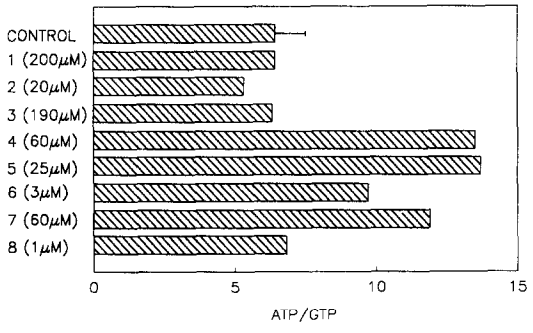

Fig. 6. Ratio of ATP to GTP in K562 cells treated with compounds **1–8**. K562 cells were incubated with compounds (the numerals refer to the compounds shown in Figs 1 and 2) at the concentrations given in parentheses for 5 days. Cells were extracted and analyzed by anion-exchange high pressure liquid chromatography as described in Materials and Methods. The control value is the mean ± SD, N = 14.

with the structural analogs, compounds **4** (ribavirin), **5** (tiazofurin), **6** (selenazofurin), and **7** (bredinin) (Fig. 2) which are inhibitors of IMP dehydrogenase [3]. The inhibition of IMP dehydrogenase by compounds **4–7** caused a depletion of guanine nucleotides as detected by an elevation of the intracellular ratio of ATP to GTP to approximately 12 from a control value of 6 (Fig. 6). A blockade at the IMP dehydrogenase step was also demonstrated by the appearance of a greater amount of newly formed purine as adenylates compared to guanylates as well as the increased release by cells of newly synthesized purines when the *de novo* biosynthesis of purines was measured by the incorporation of [^{14}C]formate (Table 1). As shown in other cellular systems [12, 13], inhibition of IMP dehydrogenase causes an increased concentration of IMP, an increased degradation of IMP to hypoxanthine, and an increased release of hypoxanthine from the cells into the surrounding medium. The measurement of IMP dehydrogenase activity in intact K562 cells by the release of tritium from [2,8-^{3}H]inosine [14, 15] also demonstrated that compounds **4–7** inhibited this enzyme (Fig. 7). In marked contrast to the IMP dehydrogenase inhibitors described above, compounds **1–3** and pyrazofurin, **8**, did not affect the ATP to GTP ratio in K562 cells (Fig. 6), did not increase release of newly synthesized purine or alter the ratio of adenylate to guanylate synthesized by the *de novo* pathway (Table 1), and did not inhibit the release of tritium from [2,8-^{3}H]inosine (Fig. 7). The absence of an effect on purine metabolism using three separate methods demonstrated that compounds **1–3** did not inhibit IMP dehydrogenase, but instead acted purely as inhibitors of pyrimidine biosynthesis.

The dichotomy between inhibition of purine or pyrimidine biosynthesis is not absolute as bredinin, **7**, caused both the release of orotic acid (Fig. 4) and

Table 1. Effects of compounds on formate incorporation

Compound	Total purine synthesized* (% of control)	Purine released into medium (% of total)	A/G ratio in cells‡
Control	100 ± 8	7.7 ± 1.6	1.7 ± 0.4
1 (200 μM)	117	6.2	1.1
2 (20 μM)	113 ± 6	5.0 ± 1.5	1.4
3 (190 μM)	86 ± 4	7.1 ± 1	1.6 ± 0.2
4 (50 μM)	73	18.6	8.1
5 (30 μM)	105	17.8	4.0
6 (4 μM)	103	27.0	6.6
7 (60 μM)	80	20.5	4.8
8 (0.4 μM)	77	10.1	2.0
9 (100 μM)	28 ± 4	14 ± 3	1.1 ± 0.1

K562 cells were incubated with the compounds (the numerals refer to the compounds shown in Figs 1 and 2) used at the concentrations shown in parentheses. K562 cells were incubated with the compounds for 20 hr followed by incubation with [^{14}C]formate for 1 hr. Separation into cell and medium fractions and measurement of radioactivity are described in Materials and Methods. The values in the table are derived from the radioactivity of various purine fractions that had incorporated ^{14}C from [^{14}C]formate and, thus, represent purines synthesized by the *de novo* pathway. The values shown are representative single determinations or means ± the deviation of two determinations at the concentrations of compounds indicated. Additional determinations (1–3) at other concentrations gave results that were consistent with the values shown.

* The total radioactive purine was obtained by adding the radioactivity of the purines in the cell and medium fractions. The value for each treatment with a compound is given as a percentage of the value for the control treatment of incubation in medium alone (18,040 cpm).

† The radioactivity of purines in the medium fraction for each treatment with a compound is given as a percentage of the total radioactive purine synthesized for treatment with the same compound.

‡ The ratio of radioactivities of adenylates and guanylates in the cell fraction.

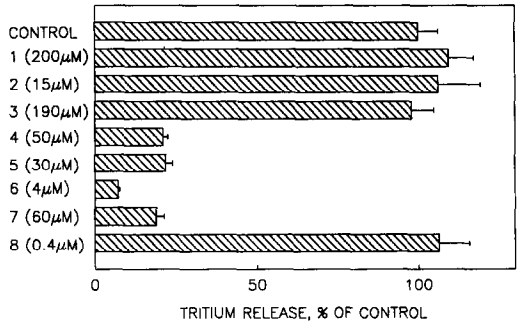

Fig. 7. Effects of compounds **1–8** on release of tritium from [^{3}H]inosine. K562 cells were treated with compounds (the numerals refer to the compounds shown in Figs 1 and 2) at the concentrations given in parentheses for 24 hr; then the release of tritium from [^{3}H]inosine was measured by the procedure of Kalman and Hsiao [15] as described in Materials and Methods. The values are the means ± SD of duplicate determinations. The control value of tritium release was 2.11×10^5 cpm.

an elevation of the ATP to GTP ratio (Fig. 6). Thus, bredinin seems to be simultaneously an inhibitor of IMP dehydrogenase and an inhibitor of OMP decarboxylase.

The pyrrole dicarboxamide, **9**, may represent another class of inhibitors. Although similar in structure to compounds **2** and **3**, compound **9** did not cause cells to accumulate [^{14}C]carbamylaspartate during incubation with [^{14}C]bicarbonate. This implies that compound **9** is not an inhibitor of pyrimidine metabolism. Nor did compound **9** cause an increase in the ratio of ATP to GTP (data not shown). This implies that compound **9** is not an inhibitor of IMP dehydrogenase. However, at concentrations above its IC_{50}, compound **9** inhibited purine *de novo* synthesis at a step preceding IMP (Table 1). The exact site of action is currently under investigation.

Growth inhibition and cell cycle arrest. Compounds **1** and **3** were very weak inhibitors of cell growth, whereas compound **2** produced stronger growth inhibition as indicated by the IC_{50} values, the micromolar concentration of compound that inhibits growth to 50% of the value for untreated cells (Table 2). For compounds **1** and **3–5**, growth inhibition was apparent 24 hr after the compounds were added, and the cell viability remained at greater than 90% for at least 4 days in the presence of the compounds at concentrations equal to the IC_{50} values. When the cells were incubated with these compounds for 4 days and then resuspended in fresh medium, the growth rate of the cells rapidly returned to normal. Therefore, these compounds were cytostatic, rather than cytolytic.

The reversal of growth inhibition by nucleosides was consistent with the proposed mechanisms of action of the compounds. Guanosine plus adenine could reverse the growth inhibition by the IMP dehydrogenase inhibitors, ribavirin and tiazofurin,

Table 2. Growth inhibition and hemoglobin production

Compound	IC_{50} (μM)	Hemoglobin-containing cells (% of total at concentration)	
1	>200	15–53%	140–200 μM
2	26 ± 9	16–62%	15–100 μM
3	160 ± 40	27–62%	100–200 μM
4	49 ± 11	47–98%	40–100 μM
5	32 ± 5	28–70%	10–60 μM
6	3 ± 1	33–70%	1–10 μM
7	60	34–89%	40–100 μM
8	0.6 ± 0.4	52–79%	0.1–1 μM
9	32 ± 8	9–52%	30–120 μM

K562 cells were incubated with the compounds (the numerals refer to the compounds shown in Figs 1 and 2) at a series of concentrations between 0.01 and 200 μM. After 5 days the cell densities were determined, and the concentration of compound that had produced a cell density that was 50% of the value for untreated, control cells was called the IC_{50}. The values are the means ± SD of results from two to seven experiments for each compound. For compound 7, a single growth versus concentration graph was used to derive the IC_{50} value. The presence of hemoglobin in the K562 cells was determined by staining with tetramethylbenzidine, as described in Materials and Methods. There was day-to-day variability in the staining, and the results from five or more determinations over a period of 1 year fell within the ranges indicated.

Table 3. Reversal of growth inhibition with nucleosides

Compound	Percent of control growth		
	No addition	+ Guo + Ade	+ Urd
Medium control	100 ± 3	90 ± 4	96 ± 3
1 (200 μM)	39 ± 1	22 ± 1	93 ± 6
2 (30 μM)	31 ± 2	21 ± 1	65 ± 2
3 (190 μM)	26 ± 1	20 ± 1	67 ± 2
4 (50 μM)	35 ± 1	91 ± 3	35 ± 1
5 (30 μM)	19 ± 1	93 ± 3	24 ± 1

K562 cells were incubated with the compounds (the numerals refer to the compounds shown in Figs 1 and 2) at the concentrations shown in parentheses in the presence or absence of 100 μM guanosine, 100 μM adenine, or 100 μM uridine where indicated for 3 days. Cell densities were determined with a Coulter Counter. Values are means ± SD for quadruplicate cultures. Starting cell density: 1×10^5 cells/mL; final control cell density: 8.1×10^5 cells/mL.

but had no effect on the growth inhibition by compounds **2** or **3** (Table 3). Adenine was required because if guanosine alone were added, the GMP formed would inhibit purine *de novo* synthesis leading to the adenylates. On the other hand, uridine could partially reverse the growth inhibition caused by the inhibitors of pyrimidine biosynthesis, compounds **2** and **3**, but had no effect on the growth inhibition caused by ribavirin or tiazofurin.

Flow cytometry was used to determine the effects of compounds **1**, **3**, **4**, and **5** on the proportion of K562 cells in different phases of the cell cycle. In 24 hr (approximately equal to the doubling time of the cells), compounds **1** and **3** produced a marked increase in the proportion of cells in S-phase (Fig. 8A and B). On the other hand, ribavirin and tiazofurin produced a broadening of the G_0–G_1 peak and a decrease in the proportion of cells in the G_2/M fraction (Fig. 8C and D).

Induction of hemoglobin synthesis. The induction of the synthesis of hemoglobin by K562 cells has been used as an *in vitro* model for hematopoietic differentiation [16, 20]. When compounds **1–3** were incubated with K562 cells for 5 days, a significant percentage of the cells stained positively for hemoglobin (Table 2). Neither orotidine nor orotic acid induced hemoglobin synthesis in K562 cells. Compounds **1–3** also induced the production of superoxide in HL-60 cells (data not shown).

DISCUSSION

The nucleosides shown in Figs 1 and 2 have the structural similarities of a five-membered heterocyclic ring and carboxamide substituents. The results presented here show that the biochemical sites of action were quite different and fell into two categories.

Compounds **1**, **2**, and **3** were inhibitors of pyrimidine biosynthesis as shown by the release of orotate, the decrease in UTP synthesis, and the accumulation of carbamylaspartate and orotidine in treated cells. The pattern of overproduction of metabolic intermediates suggests a blockade at either orotate phosphoribosyltransferase or OMP decarboxylase and a loss of feedback inhibition of the initial reactions of the path by the end products. Compounds **7** (bredinin) and **8** (pyrazofurin) also behaved as pyrimidine biosynthesis inhibitors. The fact that pyrazofurin is a known inhibitor of OMP decarboxylase [19] and that 6-azauridine, another known inhibitor of OMP decarboxylase, gave a similar [^{14}C]bicarbonate labeling profile in Ehrlich ascites cells [21], suggests that compounds **1**, **2**, and **3** also inhibited the OMP decarboxylase reaction.

Compounds **4** (ribavirin), **5** (tiazofurin), and **6** (selenazofurin) are known to be inhibitors of purine metabolism at the IMP dehydrogenase step [3]. The results for ATP to GTP ratios, tritium release from

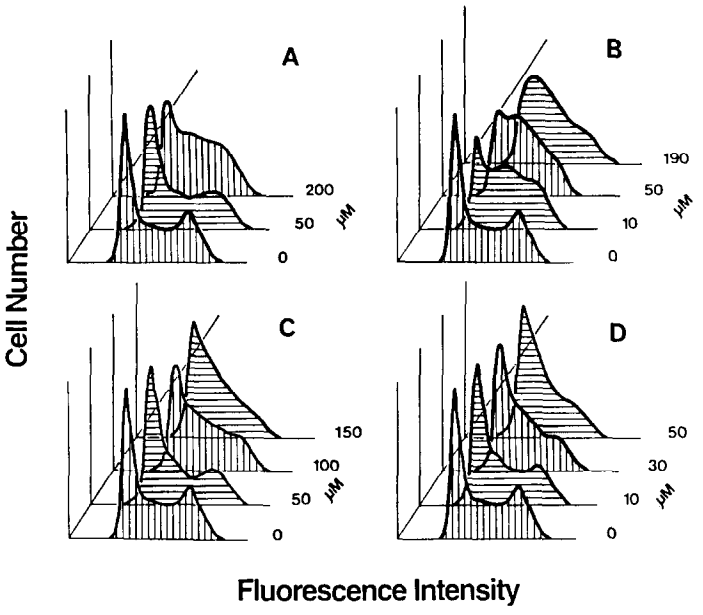

Fig. 8. Effect of inhibitors of purine and pyrimidine metabolism on the cell cycle. K562 cells were incubated with compound **1** (A), compound **3** (B), compound **4** (C), or compound **5** (D) at the indicated concentrations for 24 hr. DNA histograms were determined by flow cytometry, as described in Materials and Methods.

[^{3}H]inosine, and [^{14}C]formate incorporation are consistent with IMP dehydrogenase inhibition by compounds **4**, **5**, and **6** and, more importantly, the comparative results served to rule out IMP dehydrogenase inhibition for compounds **1**, **2**, **3**, and **9**.

The different mechanisms of action resulted in different responses at the cellular level. The inhibitors of pyrimidine metabolism caused cells to accumulate in S-phase and the inhibitors of purine metabolism caused cells to accumulate in the G_0–G_1 phase of the cell cycle. This implies that some cell cycle-dependent processes have different requirements for pyrimidine versus purine nucleotides. Since equal amounts of purines and pyrimidines are needed for DNA and RNA synthesis, some other cellular processes must be responsible for the difference in cell cycle distributions with purine versus pyrimidine biosynthesis inhibitors. The release of large amounts of orotate by cells treated with growth inhibitory and sub-growth inhibitory concentrations of compounds **1**, **2**, and **3** may have resulted from the complex regulatory nature of the pyrimidine biosynthetic pathway [22]. The sub-growth inhibitory concentrations of compounds **1**, **2**, and **3** may have inhibited OMP decarboxylase to an extent sufficient to reduce the feedback inhibition of the initial reactions of pyrimidine *de novo* synthesis by UTP and CTP and yet still allow enough nucleotide synthesis for cell growth. To complement the many reports of the *in vitro* induction of myeloid leukemia cell differentiation by ribavirin (**4**) and tiazofurin (**5**) [20, 23–29], the present results show that differentiation may also be induced by an inhibition of pyrimidine nucleotide biosynthesis. For every compound tested, there was a correlation between the concentrations that gave 50% growth inhibition and the concentrations that gave maximal differentiation (Table 2). This correlation is consistent with the hypothesis that differentiation in this *in vitro* model resulted from an inhibition of cell proliferation, rather than from a single, specific block in metabolism [30]. In conclusion, these results demonstrate the rich variety of effects on cellular metabolism produced by the five-membered ring heterocycles with a carboxamide substituent. The novel compounds characterized here may provide additional tools for the study of the *de novo* pyrimidine pathway [31].

Acknowledgements—We would like to thank Josephine Cheng, Chau Ton-That, Gina Whalin, Timothy Breen and George Chuang for excellent technical assistance.

REFERENCES

1. Harris S and Robins RK, Ribavirin: structure and antiviral activity relationships. In: *Ribavirin, A Broad Spectrum Antiviral Agent* (Eds. Smith RA and Kirkpatrick W), pp. 1–21. Academic Press, New York, 1980.
2. Tricot GJ, Jayaram HN, Nichols CR, Pennington CR, Lapis E, Weber G and Hoffman R, Hematological and biochemical action of tiazofurin (NSC 2986193) in a case of refractory acute myeloid leukemia. *Cancer Res* **47**: 4988–4991, 1987.
3. Robins RK, Revankar GR, McKernan PA, Murry BK, Kirsi JJ and North JA, The importance of IMP dehydrogenase inhibition in the broad spectrum antiviral activity of ribavirin and selenazofurin. *Adv Enzyme Regul* **24**: 29–43, 1986.
4. Poonian MS and Nowoswiat EF, Novel procedure for the synthesis of C-nucleoside analogs. Synthesis of the C-nucleoside analogs of ribavirin, bredinin, and related compounds. *J Org Chem* **45**: 203–208, 1980.
5. Witkowski JT, Robins RK, Sidwell RW and Simon LN, Design, synthesis, and broad-spectrum antiviral

activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J Med Chem* **15**: 1150–1154, 1972.
6. Srivastava PC, Pickering MV, Allen LB, Streeter DG, Campbell MT, Witkowski JT, Sidwell RW and Robins RK, Synthesis and antiviral activity of certain thiazole C-nucleosides. *J Med Chem* **20**: 256–262, 1977.
7. Srivastava PC and Robins RK, Synthesis and antitumor activity of 2-β-D-ribofuranosylselenazole-4-carboxamide and related derivatives. *J Med Chem* **26**: 445–448, 1983.
8. Mizuno K, Tsujino M, Takada M, Hayashi M, Atsumi K, Asano K and Matsuda T, Studies on bredinin. I. Isolation, characterization, and biological properties. *J Antibiot (Tokyo)* **27**: 775–782, 1974.
9. Gutowski GE, Sweeney MJ, DeLong DC, Hamill RL, Gerzon K and Dyke RW, Biochemistry and biological effects of the pyrazofurins (pyrazomycins): initial clinical trial. *Ann NY Acad Sci* **255**: 544–551, 1975.
10. Sanghvi YS, Bhattacharya BK, Kini GD, Matsumoto SS, Larson SB, Jolley WB, Robins RK and Revankar GR, Growth inhibition and induction of cellular differentiation of human myeloid leukemia cells in culture by carbamoyl congeners of ribavirin. *J Med Chem,* in press.
11. Khym JX, An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchanges. *Clin Chem* **21**: 1245–1252, 1975.
12. Hershfield MS and Seegmiller JE, Regulation of *de novo* purine synthesis in human lymphoblasts. *J Biol Chem* **251**: 7348–7354, 1976.
13. Willis RC and Seegmiller JE, Increase in purine excretion and rate of synthesis by drugs inhibiting IMP dehydrogenase or adenylosuccinate synthetase activities. *Adv Exp Med Biol* **122B**: 237–241, 1979.
14. Yalowich JC and Kalman TI, Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells *in vitro*. *Biochem Pharmacol* **34**: 2319–2324, 1985.
15. Kalman TI and Hsiao MC, Rapid measurement of IMP dehydrogenase and its inhibition in intact L1210 leukemia cells. *Proc Am Assoc Cancer Res* **28**: 16, 1987.
16. Hoffman R, Murnane MJ, Benz EJ, Prohaska R, Floyd V, Dainiak N, Forget BG and Furthmayr H, Induction of erythropoietic colonies in a human chronic myelogenous leukemia cell line. *Blood* **54**: 1182–1187, 1979.
17. Willis RC, Carlson DA and Seegmiller JE, Adenosine kinase initiates the major route of ribavirin activation in a cultured human cell line. *Proc Natl Acad Sci USA* **75**: 3042–3044, 1978.
18. Fridland A, Connelly MC and Robbins TJ, Tiazofurin metabolism in human lymphoblastoid cells: evidence for phosphorylation by adenosine kinase and 5′-nucleotidase. *Cancer Res* **46**: 532–537, 1986.
19. Dix DE, Lehman CP, Jakubowski A, Moyer JD and Handschumacher RE, Pyrazofurin metabolism, enzyme inhibition, and resistance in L5178Y cells. *Cancer Res* **39**: 4485–4490, 1979.
20. Bodner AJ, Ting RC and Gallo RC, Induction of differentiation of human promyelocytic leukemia cells (HL-60) by nucleosides and methotrexate. *J Natl Cancer Inst* **67**: 1025–1030, 1981.
21. Chen JJ and Jones ME, Effect of 6-azauridine on *de novo* pyrimidine biosynthesis in cultured Ehrlich ascites cells. *J Biol Chem* **254**: 4908–4914, 1979.
22. Jones ME, Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. *Annu Rev Biochem* **49**: 253–279, 1980.
23. Sokoloski JA, Blair OC and Sartorelli AC, Alterations in glycoprotein synthesis and guanosine triphosphate levels associated with the differentiation of HL-60 leukemia cells produced by inhibitors of inosine 5′-phosphate dehydrogenase. *Cancer Res* **46**: 2314–2319, 1986.
24. Kerr SJ, Ribavirin induced differentiation of murine erythroleukemia cells. *Mol Cell Biochem* **77**: 187–194, 1987.
25. Olah E, Natsumeda Y, Ikegami T, Kote Z, Horanyi M, Szelenyi J, Paulik E, Kremmer T, Hollan SR, Sugar J and Weber G, Induction of erythroid differentiation and modulation of gene expression by tiazofurin in K-562 leukemia cells. *Proc Natl Acad Sci USA* **85**: 6533–6537, 1988.
26. Yu AL, Connor JD, Page T, Lemas V and Yu J, Induction of hemoglobin accumulation in human K562 cells by inhibitors of inosine monophosphate dehydrogenase. *Proc Am Assoc Cancer Res* **29**: 32, 1988.
27. Lucas DL, Robins RK, Knight RD and Wright DG, Induced maturation of the human promyelocytic leukemia cell line, HL-60, by 2-β-D-ribofuranosylselenazole-4-carboxamide. *Biochem Biophys Res Commun* **115**: 971–980, 1983.
28. Lucas DL, Webster HK and Wright DG, Purine metabolism in myeloid precursor cells during maturation. Studies with the HL-60 cell line. *J Clin Invest* **72**: 1889–1900, 1983.
29. Kharbanda SM, Sherman ML, Spriggs DR and Kufe DW, Effects of tiazofurin on proton-oncogene expression during HL-60 cell differentiation. *Cancer Res* **48**: 5965–5968, 1988.
30. Bloch A, Induced cell differentiation in cancer therapy. *Cancer Treat Rep* **68**: 199–205, 1984.
31. Kensler TW and Cooney DA, Chemotherapeutic inhibitors of the enzymes of the *de novo* pyrimidine pathway. *Adv Pharmacol Chemother* **18**: 273–352, 1981.