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INHIBITION OF OROTIDYLATE DECARBOXYLASE BY 4(5*H*)-OXO-1-β-D-RIBOFURANOSYLPYRAZOLO[3,4-*d*] PYRIMIDINE-3-THIOCARBOXAMIDE (APR-TC) IN B LYMPHOBLASTS

ACTIVATION BY ADENOSINE KINASE

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Abstract—The nucleoside allopurinol riboside-3-thiocarboxamide (APR-TC; 4-(5*H*)oxo-1-β-D-ribofuranosylpyrazolo[3,4,*d*]pyrimidine-3-thiocarboxamide) demonstrates potent *in vitro* antiviral activity against various DNA and RNA viruses and cytostatic activity against a variety of cell lines in culture. The IC_{50} for APR-TC in the splenic derived B lymphoblast cell line, WI-L2, was 0.3 μM. Adenosine kinase-deficient WI-L2 cells were resistant to growth inhibition by APR-TC, indicating that adenosine kinase (EC 2.7.1.20) is responsible for phosphorylation of APR-TC to form the monophosphate derivative (APR-TC-5'P). A 4-hr incubation of cells with 50 μM APR-TC resulted in severe depletion of intracellular pyrimidine nucleotide pools and the accumulation of 3 μM APR-TC-5'P. The cytotoxicity of APR-TC was reversed by uridine, indicating that the active form of this compound inhibits the *de novo* pyrimidine biosynthetic pathway. Further, APR-TC-treated cells could not utilize the pyrimidine nucleotide precursor [6-^{14}C]orotic acid, suggesting that the UMP synthase complex is the major cellular site of inhibition. In studies utilizing cell-free lysates of WI-L2, chemically prepared APR-TC-5'P provided potent inhibition of the orotidylate decarboxylase activity (ODCase, EC 4.1.1.23) of the UMP synthase complex. APR-TC-5'P was competitive with OMP, and a K_i value of 0.35 nM was determined.

The UMP synthase complex [1] of mammalian cells consists of orotate phosphoribosyltransferase (OPRTase,† EC 2.4.2.10) and orotidylate decarboxylase (ODCase, EC 4.1.1.23). Together the enzymes lead to formation of UMP from orotate and 5-phosphoribosyl-1-pyrophosphate (PRPP) with the transient intermediate formation of orotidine 5'-monophosphate [2]. Inhibitors of the ODCase activity of the complex have continued potential use as chemotherapeutic agents and as probes for characterization of the enzyme active site [see Refs. 3 and 4 for reviews]. 6-Azauridine [5] and pyrazofurin [6], for example, inhibit ODCase and also exhibit antiviral and antitumor activity. ODCase inhibitors are reportedly effective in the treatment of polycythemia vera, mycosis fungoides and psoriasis [7]. Other compounds such as 6-hydroxyuridine [8] and 1-oxipurinol riboside (OPR) [9] as their respective monophosphates have shown potent inhibition against cell-free ODCase from various sources and have been of value in enzymatic studies, but they are devoid of chemotherapeutic activity [10, 11]. Allopurinol riboside-3-thiocarboxamide (APR-TC) was prepared previously [12] and was identified as a potent antiviral agent with broad *in vitro* activity against a range of DNA and RNA viruses [13]. APR-TC also inhibited the proliferation of a variety of cell lines including the B lymphoblast-derived WI-L2 cell line (IC_{50} = 0.3 μM). We have studied the effect of APR-TC on WI-L2 cells and certain derivative cell lines and report here that the 5'-monophosphate derivative of APR-TC was a potent inhibitor of the ODCase activity of the UMP synthase complex. The bulk tolerance by ODCase for modification at the 3-position of pyrazolo[3,4-*d*]pyrimidine nucleoside 5'-monophosphates has not been recognized previously.

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† Abbreviations: OPRTase, orotate phosphoribosyltransferase (EC 2.4.2.10); ODCase, orotidylate decarboxylase (EC 4.1.1.23); APR-TC, allopurinol riboside-3-thiocarboxamide (4-(5*H*)oxo-1-β-D-ribofuranosylpyrazolo-[3,4,*d*]pyrimidine-3-thiocarboxamide, 1-β-D-ribofuranosylpyrazolo[3,4-*d*]-4-pyrimidinone-3-thiocarboxamide); APR-TC-5'P, allopurinol riboside-3-thiocarboxamide 5'-monophosphate; PF, pyrazofurin; 6-azaUrd, 6-azauridine; 6-azaUMP, 6-azauridine 5'-monophosphate; OPR, 1-oxipurinol riboside (1-β-D-ribofuranosylpyrazolo[3,4-*d*]-pyrimidine-4,6-dione); OPR-5'P, 1-oxipurinol riboside 5'-monophosphate; AKase, adenosine kinase (EC 2.7.1.20); AK$^-$, adenosine kinase-deficient WI-L2 cells; HPRTase, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); HPRT$^-$, hypoxanthine phosphoribosyltransferase-deficient WI-L2 cells; CAA, carbamyl aspartic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; and SAX-HPLC, strong anion exchange-high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials. RPMI 1640 medium and fetal bovine

serum were purchased from Flow Laboratories, Inc. [6-^{14}C]Orotate and [7-^{14}C]orotidine 5′-monophosphate were from New England Nuclear. 6-Azauridine was from Calbiochem. APR-TC was synthesized as previously described [12].

Chemical synthesis of APR-TC-5′P. To a suspension of APR-TC (1.0 g, 3.0 mmol) in trimethyl phosphate (50 ml) cooled to 0° was added phosphorus oxychloride (0.36 ml, 3.9 mmol). The reaction mixture was stirred at 0–5° for 48 hr after which time the yellow homogeneous solution was poured over ice (100 ml) and stirred vigorously. The mixture was extracted with chloroform (3 × 50 ml) to remove the trimethyl phosphate. The residual chloroform in the aqueous layer was removed *in vacuo*, and the pH was adjusted to 7.0 with solid NH_4HCO_3. The solution was loaded onto a DEAE-Sephadex column (Sigma, HCO_3^- form, 20 × 3.2 cm) and eluted with a linear ammonium bicarbonate gradient (0–600 mM NH_4HCO_3, 6 liters total volume). The appropriate fractions were pooled and repeatedly lyophilized. The residue was dried *in vacuo* over P_2O_5 with gentle heating (refluxing acetone) to yield 530 mg (40%) of the monoammonium salt as a yellow powder. ^{1}H-NMR (DMSO-d_6): δ 3.72 (m, 1), 3.89 (m, 1), 4.08 (q, 1, J = 6.0 Hz), 4.33 (t, 1, J = 5.1 Hz), 4.57 (t, 1, J = 4.8 Hz), 6.11 (d, 1, J = 3.9 Hz, C_1,*H*), 8.27 (s, 1, C_6*H*, 10.22 and 11.36 (2s, 2, CSN*H*$_2$), 11.36 (s, 1). Anal. Calcd. for $C_{11}H_{17}N_6O_8PS$ 1.25 H_2O: C, 29.57; H, 4.40; N, 18.81; P, 6.93; S, 7.17. Found: C, 29.94; H, 4.25; N, 18.38, P, 6.61; S, 7.13.

Cell lines. The B cell line, WI-L2, and derivatives have been described previously [14]. WI-L2 is the normal lymphoblast phenotype. The enzyme-deficient cell lines selected *in vitro* are: HPRT$^-$, a hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)-deficient line and AK$^-$, an adenosine kinase (EC 2.7.1.20)-deficient line. Cells were cultured (37°, 5% CO_2) in RPMI-1640 medium containing 5% dialyzed fetal bovine serum, 20 mM sodium HEPES, pH 7.5, and 2 mM glutamine and maintained in log phase growth between 0.5 and 12 × 10^5 cells/ml. The mutant cell lines were periodically reselected by treatment with 6-thioguanine (HPRT$^-$) or tubercidin (AK$^-$).

Cellular nucleoside/nucleotide pool preparation. Samples for nucleotide analysis and nucleoside/base analysis of cells and medium, respectively, were prepared [15] by harvesting 15- to 20-ml suspension cultures of WI-L2 cells at a cell density of 5–10 × 10^5 cells/ml. Cells were collected by centrifugation (1500 *g*, 5 min). The medium was transferred to an iced tube containing 0.1 vol. of 4.0 N perchloric acid. The cell pellet was resuspended in cold 0.4 N $HClO_4$ (100 μl/2–6 × 10^6 cells). After vortexing for 1 min, the precipitated material in each case was removed by centrifugation, and the resulting supernatant liquid was neutralized with tri-*n*-octylamine (Aldrich)/1,1,2-trichlorotriflouroethane (Freon-113, Matheson) as described [16] and stored at −70° until analysis by high pressure liquid chromatography.

HPLC analysis. Sample components were separated with an LKB model 2150 gradient HPLC system at ambient temperature on a Partisil PXS 10/25 SAX column (Whatman) by elution with a gradient formed with buffer A (10 mM potassium phosphate, pH 3.83) and buffer B (0.35 M potassium phosphate, 0.5 M KCl, pH 3.45) with a combined flow rate of 1.5 ml/min as follows: 5 min of buffer A; 6 min of linear increase to 30% buffer B; 1.5 min of linear increase to 60% buffer B; 2 min of linear increase to 100% buffer B; 17.5 min at 100% buffer B. UV absorbance was monitored with either a Kratos model 757 variable wavelength detector or an LKB model 2140 diode array detector and a Ramona-D radiometric detector when monitoring elution of radiolabeled compounds. Nucleoside/base analysis was performed on samples of neutralized medium by chromatography at ambient temperature on an Altex Ultrasphere-ODS reverse phase column (Beckman) developed with a linear gradient of buffer A (10 mM KPO_4, pH 3.83) to 20% component C (60% aqueous CH_3CN) at a combined flow rate of 1.0 ml/min over 15 min.

Mutant toxicity assay. Cultures of WI-L2 cells, HPRTase- or AKase-deficient WI-L2 cells (1–2 × 10^4 cells/100 μl well) were established in 96-well microtiter plates. After a 4-hr preliminary incubation, graded concentrations of drug were added and incubation was continued for 64–72 hr. Cell growth was determined by means of an electronic particle counter (Coulter counter model ZBI) or by the MTT dye reduction method [17]. For the dye reduction assay cell cultures were incubated with 25 μl of 3(4,5-*d*-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma; 1 mg/ml in Dulbecco's phosphate-buffered saline) for 2–3 hr at 37°. The resulting formazan precipitate was solubilized with 200 μl of 0.04 N HCl in isopropyl alcohol. Absorbance was read in a Titertek multiscan MCC scanning well spectrophotometer (Flow Laboratories, Inc., McLean, VA) at a test wavelength of 540 nm and a reference wavelength of 620 nm. Cell growth is directly proportional to absorbance. Reversal of APR-TC cytotoxicity was performed by supplementing WI-L2 or AK$^-$ cultures with combinations of drug and/or uridine. Cell growth was determined by the MTT dye reduction assay as described above.

Enzyme assays. Cell-free lysates were prepared as follows: WI-L2 cultures (1 × 10^6 cells/ml) were harvested and cells were washed twice with Hanks' buffer by centrifugation at 1500 *g* for 5 min and resuspension. The final cell pellet was resuspended to 5 × 10^7 cells/ml in a buffer composed of 10 mM Tris–Cl, 0.5 mM sodium EDTA and 2 mM dithiothreitol and lysed by three cycles of freezing (liquid N_2) and thawing at 37°. The suspension was centrifuged at 100,000 *g* for 60 min, and the supernatant fraction was used immediately or stored at −70° until use. Orotidylate decarboxylase activity was assayed as previously described [1] by measuring the release of $^{14}CO_2$ [18] from [7-^{14}C]orotidine 5′-monophosphate (OMP). ODCase activity was measured at pH 7.5, 37°, in a final volume of 0.5 ml containing 20 mM Tris–Cl, 2 mM dithiothreitol, 0.1 mM EDTA and 40 μM [7-^{14}C]OMP (1.0 Ci/mol). The concentration of OMP was determined at 267 nm using a molar extinction coefficient of 9420 [19]. Adenosine kinase activity was measured by the filter binding assay method [20]. Cellular activities were determined at 37° with an assay mixture con-

taining 4 mM ATP, 1.5 mM $MgCl_2$, 50 μM [8-^{14}C]adenosine (50 mCi/mmol) and 100 mM Tris-maleate, pH 5.5. The enzymatic breakdown of adenosine was prevented by the addition of *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) to the cell-free lysate to give a 5 μM final concentration. Assays were started by the addition of protein. Inhibition studies were performed as above, but with 10 μM adenosine and drug at 1 mM. K_i determinations utilized [8-^{14}C]adenosine at concentrations of either 5 or 10 μM. The amount of enzyme, time of assay, and sampling volume were adjusted to give acceptable conversion of [8-^{14}C]adenosine to [8-^{14}C]AMP. Each DE-81 filter was spotted with a constant sample volume and immersed in distilled water (4 liters) to terminate the reaction. Filters were washed three times with water and once with 95% ethanol (100 ml). Dry filters were placed in scintillation vials, and radioactivity was determined in 10 ml of toluene-based scintillation fluid. Hypoxanthine phosphoribosyltransferase activity was determined with protein from WI-L2 lysate which was prepared without dithiothreitol. Assays contained 1 mM phosphoribosylpyrophosphate, 5 mM $MgCl_2$, 50 mM Tris–Cl, pH 7.5, and 4 μM [8-^{14}C]hypoxanthine. Activity was quantitated by the filter binding method as described for adenosine kinase. Protein was determined by the method of Bradford [21].

[6-^{14}C]*Orotate labeling of UTP.* Log phase cultures of WI-L2 cells (6–10 × 10^5 cells/ml) were treated with APR-TC or with an equal volume of sterile water and incubated for 1 hr at 37° under 5% CO_2 atmosphere. [6-^{14}C]Orotate (50 Ci/mol, 10 μM final concentration) was added to each flask and incubation was continued for 1.5 hr. Intracellular pools were prepared as described above and analyzed by SAX-HPLC. Eluants were monitored by UV (254 nm) and by radiometric detection.

[^{14}C]*Bicarbonate labeling of cellular nucleotides.* Cultures of WI-L2 cells (20 ml at 4–6 × 10^5 cells/ml) were incubated with various concentrations of compounds for 3 hr at 37°. Cells were collected by centrifugation and the supernatant fraction was removed. The resulting cell pellet was resuspended in one-third volume of bicarbonate-free RPMI-1640 medium containing compound at the same final concentration. [^{14}C]Bicarbonate (20 μl; 56 Ci/mol) was added, the tube was sealed and incubation was continued for 1 hr. The cells were collected by centrifugation and the medium was removed. Cellular nucleotide pools were prepared as described above.

Continuous culture of WI-L2 cells with APR-TC. WI-L2 cells were grown in the presence of APR-TC (0.1 μM continuously, or initially at 0.1 μM for 14 days, then continuously at 1.0 μM). Drug-treated cultures were centrifuged to pellet cells, and the pellets were washed by resuspension in fresh medium containing no drug. Cell lysates were prepared from each pellet as previously described. Enzyme activities were determined as described above.

RESULTS

Effect of APR-TC on WI-L2 and certain enzyme-deficient WI-L2 cells. Adenosine kinase was required for phosphorylation of APR-TC as demonstrated by treatment of the various enzyme-deficient WI-L2 cell lines with APR-TC (Table 1). The growth of WI-L2 and HPRT$^-$ cells was inhibited at concentrations down to 0.9 μM APR-TC, but not at 0.09 μM. The IC_{50} value was determined to be 0.3 μM for the inhibition of WI-L2 cells. The line selected for adenosine kinase deficiency, however, was not inhibited at 9.0 μM, demonstrating the requirement for functional adenosine kinase to produce the cytotoxic effect.

Table 1. Mutant toxicity assay of cell growth inhibition by APR-TC

Drug concn (μM)	Cell growth (% of control)		
	WI-L2	AK$^-$	HPRT$^-$
No drug (control)	100	100	100
9.0	10	119	12
2.5*	25	107	ND†
0.90	43	120	57
0.09	104	123	111

The lymphoblast cell lines, WI-L2, AKase. or HPRTase-deficient mutants at a density of 0.8–1.2 × 10^5 cells/ml were placed into a 96-well plate and incubated for 4 hr before addition of drug at the indicated concentrations. Control cells numbered 8–12 × 10^5 cells/ml at the conclusion of the assay. Cell growth was determined by the MTT dye reduction assay as described in Experimental Procedures.

* Cells at this concentration were counted by means of an electronic particle counter.

† Not determined.

Interaction of APR-TC with adenosine kinase. Inhibition of adenosine kinase activity was demonstrated by APR-TC. At 1 mM APR-TC, the conversion of [8-^{14}C]adenosine to [8-^{14}C]AMP occurred to 54.7% as compared to control (no drug added = 100% conversion). In contrast, an equimolar amount of pyrazofurin was only slightly inhibitory (98.3%). Adenosine at 1 mM provided less than 5% conversion. The K_i value for APR-TC was determined subsequently to be 270 μM at an apparent K_m value for adenosine of 17 μM.

Identification of APR-TC and APR-TC-5'P in intracellular nucleotide pools. WI-L2 cells incubated in the presence of APR-TC were examined for intracellular metabolites. SAX-HPLC analysis of nucleotide pools utilizing UV-diode array spectral analysis allows identification of APR-TC and APR-TC-5'P due to the UV spectrum exhibited by APR-TC [12]. The spectrum of APR-TC has λ_{max} values at 248 nm and 315 nm with an absorbance ratio 248 nm/315 nm = 0.46 (pH 3.8). The SAX-HPLC chromatogram of APR-TC-treated cells (Fig. 1) at 254 nm and 314 nm confirmed the presence of intracellular APR-TC and APR-TC-5'P. The phosphorylated species was detected at 11.5 min in the nucleoside monophosphate region of the chromatogram. Synthetically prepared APR-TC-5'P eluted between standard IMP (11.0 min) and GMP (12.2 min) and coeluted with the APR-TC-5'P peak from treated WI-L2 cell extracts. The concentration of APR-TC-5'P represented in the chromatogram

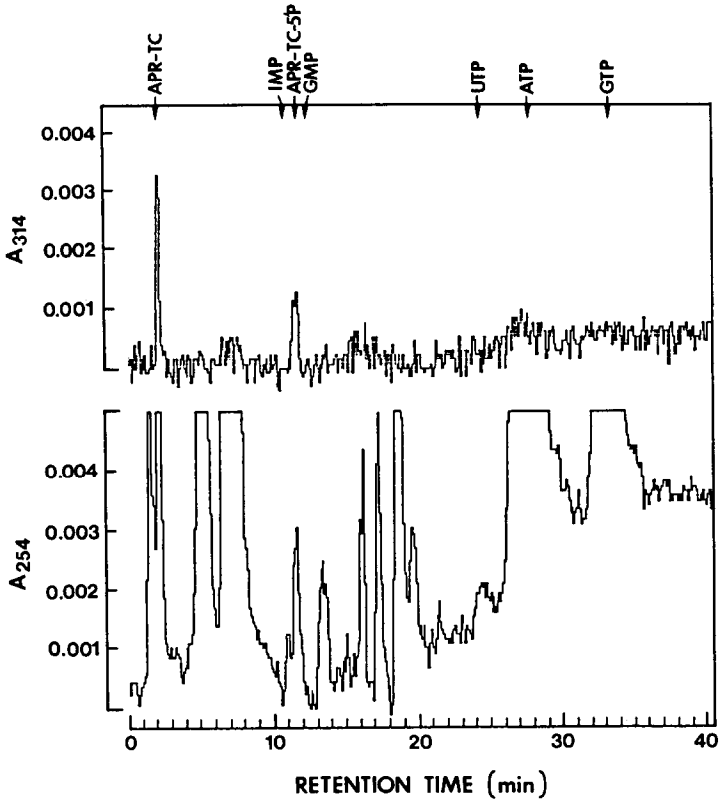

Fig. 1. SAX-HPLC chromatogram of APR-TC-treated WI-L2 cell extracts. WI-L2 cells (9×10^5 cells/ml) were treated with 50 μM APR-TC for 4 hr at 37°. Preparation of cell extracts and SAX-HPLC methods are described under Experimental Procedures. The acid-soluble fraction from 3.5×10^6 cells was analyzed at 314 nm and 254 nm by spectral diode array detection. Retention times of standards are indicated by arrows at the top of the figure.

was estimated to be 0.1 μM by comparison with standards. This corresponds to 3.0 pmol/10^6 cells. Assuming an average cell volume of 1.0 μl/10^6 cells [22], the intracellular APR-TC-5'P concentration would then be 3.3 μM. The UTP and CTP peaks were depleted by this treatment (Fig. 1).

Uridine reversal of APR-TC toxicity. The antiviral effects of APR-TC have been shown previously to be reversed by uridine [14]. The APR-TC-mediated cytotoxicity of WI-L2 cells could also be reversed as demonstrated by treatment of cells with APR-TC in the presence and absence of uridine (Table 2). Exogenous uridine is metabolized directly to UMP in the cell. Reversal of cytotoxicity by uridine indicated inhibition at a site preceding UMP in the pyrimidine *de novo* biosynthetic pathway. The inhibition of cell growth by other inhibitors of pyrimidine biosynthesis was similarly reversed (Table 2).

APR-TC inhibition of [^{14}C]orotate and [^{14}C]bicarbonate labeling of UTP. The site of *de novo* pyrimidine biosynthesis inhibition was identified as the ODCase activity by labeling cellular nucleotides with [6-^{14}C]orotate or [^{14}C]bicarbonate. WI-L2 cells normally incorporated high levels of [6-^{14}C]orotate into UTP (Fig. 2). In contrast, APR-TC-treated cells showed radioactivity only in the OMP peak, and the UTP peak was severely depleted. The acid-insoluble fractions were also analyzed, and the APR-TC-treated pellet had <8% of the orotate-dependent label of control cells (i.e. 2,000 cpm vs 26,000 cpm for control). The accumulation of OMP suggests that the OPRTase activity of the UMP synthase complex is functional in the presence of APR-TC and defines the site of inhibition to the ODCase activity. The detection of low OMP levels in APR-TC treated cells (Fig. 2) is consistent with the observation that OMP does not normally accumulate within the cell [2, 23, 24]. The accumulation of OMP and the depletion of UMP by APR-TC treatment were confirmed by using [^{14}C]bicarbonate as the tracer material to label both purine and pyrimidine nucleotides and intermediates. As shown in Table 3, APR-TC, 6-azaUrd and pyrazofurin had a similar effect on the [^{14}C]bicarbonate labeling pattern; a depletion of UTP and an accumulation of OMP and carbamoyl aspartic acid (CAA) [25]. The ATP peak was reduced to a similar extent in each of the drug-treated cell extracts.

APR-TC-5'P inhibition of orotidylate decarboxylase. The OMP concentration used to determine the activity of ODCase was 20 or 40 μM and corresponds to the "high" OMP concentration described in experiments with ODCase from yeast and rat liver [9]. The K_i value of the synthetically prepared APR-TC-5'P is shown by means of a Dixon plot (Fig. 3). The K_i value was estimated from the intersection of

Table 2. Effect of uridine on drug cytotoxicity with WI-L2 and adenosine kinase-deficient (AK^-) WI-L2 cells

Compound	Concn (μM)	Cell growth (% control*) WI-L2	AK^-
A. Without uridine			
APR-TC	25	5.5 ± 1.1	110.1 ± 1.6
	50	3.2 ± 0.4	107.5 ± 4.2
6-AzaUrd	25	6.2 ± 1.5	7.3 ± 0.5
	50	4.2 ± 0.6	5.4 ± 0.2
PF	50	4.1 ± 1.0	ND†
B. With uridine			
APR-TC	25	72.6 ± 3.0	121.8 ± 1.9
	50	58.6 ± 2.1	130.0 ± 1.6
6-AzaUrd	25	98.5 ± 1.9	122.3 ± 3.7
	50	90.6 ± 2.1	118.1 ± 2.1
PF	50	68.2 ± 3.3	ND†

Cells were treated with drug and with uridine (1.0 mM) or an equal volume of water. After 64 hr, cell growth was determined by the MTT dye reduction method. Cell growth is expressed as percent of control absorbance (mean ± SD, N = 4). PF, pyrazofurin.

* Control absorbance: WI-L2, 0.471 ± 0.008; AK^-, 0.426 ± 0.008.

† Not determined.

the linear regression lines obtained from two concentrations of OMP and gave a value of 0.35 nM. For comparison, 6-azaUMP tested in parallel displayed a K_i value of 600 nM, in agreement with reported values [26].

Enzyme activities of WI-L2 cells cultured in the presence of APR-TC. Lysates from WI-L2 cells grown continuously in the presence of APR-TC were assayed for ODCase, adenosine kinase and hypoxanthine phosphoribosyltransferase activities. These enzymes represent the inhibited activity, the APR-TC phosphorylating activity, and an enzyme not directly involved in the mechanism of inhibition of APR-TC respectively. ODCase activity increased 60% by treatment with 0.1 μM APR-TC compared to untreated cultures (see Table 4). HPRTase remained fairly constant for all samples. The adenosine kinase activity varied to the greatest extent showing an increase to over twice (216%) the control value with 0.1 μM APR-TC after 35 days of treatment. Cells incubated initially at 0.1 μM APR-TC for 2 weeks and subsequently with 1.0 μM for 25 days had reduced ODCase activity, but also had reduced levels of adenosine kinase activity (46% of control). When APR-TC (0.1 μM) treated cells were cultured in drug-free medium for a period of 14 days, the ODCase activity fell to untreated control levels (data not shown). A similar decrease of elevated ODCase activity upon release of selective pressure has been observed in rat hepatoma cells treated with pyrazofurin [27].

* Substitution of larger groups at certain positions about the heterocyclic ring may limit adenosine kinase substrate activity; however, there is a precedent for substitution of a carboxamido group at a site corresponding to the 3-position of APR-TC with negligible effect on substrate activity [30]. Thus, the substrate efficiency (relative V'_{max}/K'_m) of sangivamycin is similar to adenosine (sangivamycin = 245; adenosine = 250).

DISCUSSION

One of the most potent inhibitors of ODCase yet reported [2, 26] is 1-oxipurinol riboside 5'-monophosphate (OPR-5'P; see Fig. 4). K_i values from 2 to 20 nM have been reported for various enzyme preparations under assay conditions similar to those used in the present study [9, 26]. Xanthosine 5'-monophosphate, the naturally occurring isomer of OPR-5'-P, has been recognized as the most potent natural inhibitor of ODCase [2, 26, 28], providing a further example of accommodation of a bicyclic nucleoside monophosphate by ODCase.

APR-TC-5'P and OPR-5'P are derived from the same parent heterocyclic ring, but the substitutions are geometrically distinct. OPR-5'P possesses the 2,4-diketo structure of orotate and apparently acts as a tight-binding inhibitor [3]. APR-TC-5'P is a structural IMP analog, and the potent inhibition by APR-TC-5'P demonstrates a greater bulk tolerance by ODCase in the proximity of the C-5 position of bound OMP than has been recognized previously. It is also of interest that APR-TC, as an inosine analog, would be a substrate* for adenosine kinase since inosine is not a substrate for this enzyme [29]. However, certain inosine analogues are known substrates for adenosine kinase. Allopurinol riboside (4(5*H*)-oxo-1-β-D-ribofuranosyl[3,4-*d*]pyrimidine), for example, has been reported to have weak substrate activity with adenosine kinase (K_m = 1.3 mM) [30]. 8-Azainosine is also phosphorylated by adenosine kinase [31]. The interaction of APR-TC with adenosine kinase is unfavourable as demonstrated by a K_i value of 270 μM, and the activation of APR-TC by adenosine kinase may be the rate-limiting step in cellular ODCase inhibition.

Pyrazofurin is also phosphorylated by adenosine kinase and acts at the level of the 5'-monophosphate to inhibit orotidylate decarboxylase. However, pyrazofurin is also metabolized to higher phosphate

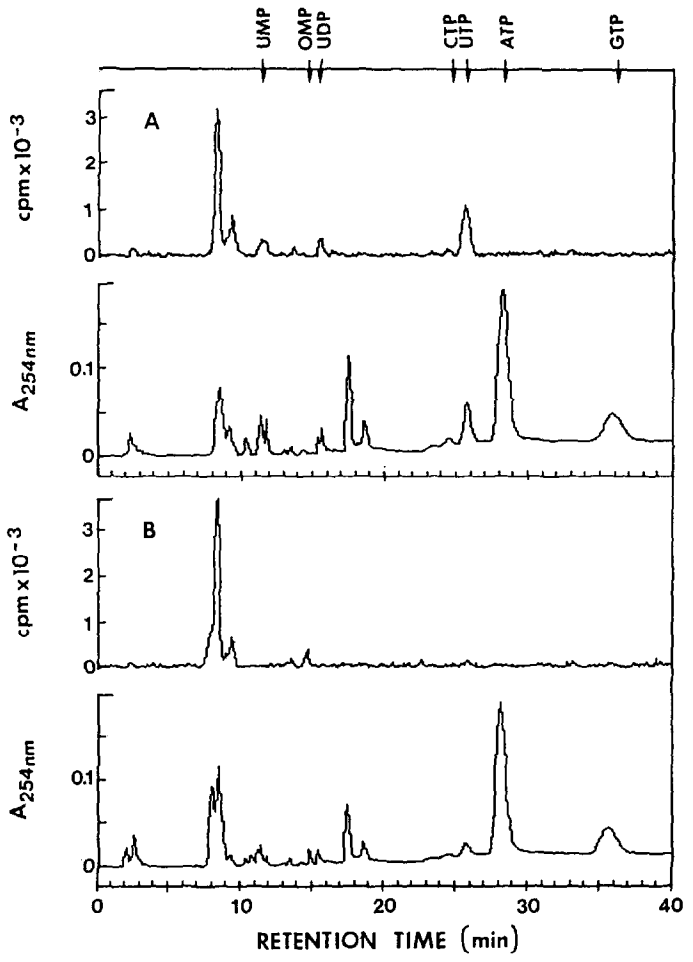

Fig. 2. SAX-HPLC chromatogram of acid-soluble extracts of WI-L2 cells treated with [6-^{14}C]orotic acid. Each chromatogram represents the acid-soluble extract of 4×10^6 cells/100 μl injection. WI-L2 cells (9×10^5 cells/ml) were preincubated with (A) sterile water or (B) 50 μM APR-TC for 1 hr before pulsing with [6-^{14}C]orotic acid (10 μM, 47 Ci/mol) for 2.5 hr. Column eluate was monitored for UV absorption at 254 nm (bottom) and for radioactivity (top) utilizing tandem in-line ultraviolet (UV) and radiometric detectors. Cell extract preparation and instrumentation are described under Experimental Procedures. Retention times of standards are indicated by arrows at the top of the figure.

Table 3. Comparative [^{14}C]bicarbonate labeling of WI-L2 cell acid-soluble pools treated with drug

Compound	Concn (μM)	Area units* CAA†	OMP	UTP	% of control	ATP	% of control
Control		ND	ND	11.30	100.0	28.96	100.0
APR-TC	0.4	44.34	2.07	0.32	2.8	22.29	76.9
PF	0.4	42.01	1.50	ND	ND	20.86	72.0
6-AzaUrd	0.6	38.04	1.23	0.55	4.8	22.10	76.3

Cells were treated with drug for 3 hr and then resuspended in bicarbonate-free RPMI-1640 medium containing APR-TC, pyrazofurin, or 6-AzaUrd. [^{14}C]Bicarbonate was added and incubation was continued for 1 hr. Acid-soluble extracts from 2×10^6 cells were separated by SAX-HPLC. ND = not detected; it represents less than 0.05 area units.

* One area unit = 2500 cpm of injected standard nucleotide.

† Carbamyl aspartic acid is a precursor in the *de novo* synthesis of UMP.

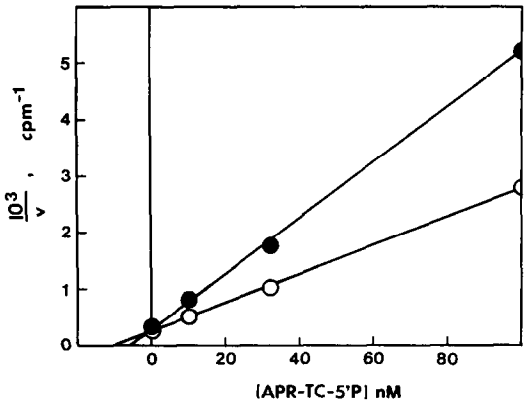

Fig. 3. APR-TC-5'P inhibition of orotidylate decarboxylase. Orotidylate decarboxylase from WI-L2 cell-free lysates was investigated for inhibition by APR-TC-5'P as described under Experimental Procedures. The K_i value for APR-TC-5'P inhibition of ODCase was determined by observing the decrease in the rate of decarboxylation of 20 (●) or 40 μM (○) [6-^{14}C]OMP (1.0 Ci/mol). The concentration of APR-TC-5'P was varied as indicated. Each point represents the average of triplicate measurements. One standard deviation unit is within the size of each graph symbol.

derivatives [32], and although ODCase inhibition appears to be the major site of action, pyrazofurin 5'-phosphate also inhibits *de novo* purine biosynthesis [33].

Kinetic parameters for ODCase from certain sources are reportedly dependent upon the state of aggregation of the UMP synthase preparation [34, 35]. The ODCase activity which was used for the present study was not characterized with regard to quarternary structure; however, the kinetic parameters were determined in the presence of dithiothreitol, which prevents aggregation of the monomer to higher molecular weight forms [36]. APR-TC-5'P was tested for inhibitory activity against WI-L2 ODCase and compared with 6-azaUMP, which is characterized with regard to ODCase inhibition in a number of systems. APR-TC-5'P was found to be 1000 times more potent than 6-azaUMP. The K_i value of 600 nM for 6-azaUrd is in agreement with values obtained with ODCase from various sources [2, 26], and this value was also reported for the low molecular weight (monomer) form of UMP synthase isolated from erythrocytes [26].

It has been suggested recently [37] that antiviral agents which are activated by an enzyme other than thymidine kinase and which are not incorporated into the viral genome should be identified. APR-TC represents such a nucleoside. APR-TC is activated by cellular adenosine kinase to form the nucleoside 5'-phosphate. At the monophosphate level, the nucleoside analog interferes with normal *de novo* UMP synthesis by potent inhibition of orotidylate decarboxylase and does demonstrate interesting antiviral activity.

As a highly potent inhibitor of orotidylate decarboxylase, APR-TC has potential utility in certain diagnostic applications, in combination with other compounds in studies of pyrimidine metabolism and as a probe of the ODCase active site. Although APR-TC is effective as an antiviral nucleoside *in vitro*, it has little chemotherapeutic effect in

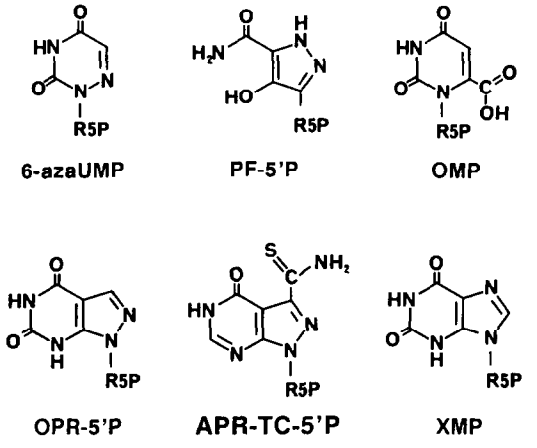

Fig. 4. Structures of OMP, APR-TC-5'P and other inhibitors of ODCase. R5P = ribose-5-phosphate.

Table 4. Relative activity of enzymes from APR-TC-treated WI-L2 cells

Time (days)	APR-TC (μM)	Enzyme activity (% of control)		
		ODCase	HPRTase	AKase
Control		100.0 ± 1.7	100.0 ± 5.9	100.0 ± 2.1
13	0.1	159.1 ± 1.9	133.8 ± 4.8	107.1 ± 5.6
35	0.1	165.6 ± 4.3	107.1 ± 6.0	215.6 ± 9.9
25	1.0	131.3 ± 2.6	108.6 ± 3.2	46.1 ± 2.1

WI-L2 cells were cultured continuously for various time periods in the presence of APR-TC. After a period of 14 days at 0.1 μM APR-TC, one culture was treated with 1.0 μM APR-TC and was subsequently cultured at 1.0 μM APR-TC for 25 days. WI-L2 lysate preparation and enzyme assays are described under Experimental Procedures. The enzyme activity of three samples (mean ± SD) is expressed as percent of control. The control levels of activity, expressed in nmol/min/mg of lysate protein, are: ODCase, 0.83 ± 0.01; HPRTase, 2.91 ± 0.17; and AKase, 0.14 ± 0.01.

mice infected with various types of virus [14]. The *in vivo* chemotherapeutic utility of APR-TC, like other specific inhibitors of pyrimidine *de novo* biosynthesis, may be limited by circulating plasma uridine which circumvents the metabolic block by supplying cells with UMP through the action of uridine kinase [38].

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