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An *in Vivo* and *in Vitro* Evaluation of 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine: An Inhibitor of Human Lymphoblast Purine Nucleoside Phosphorylase

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SUMMARY

WILLIS, R. C., R. K. ROBINS AND J. E. SEEGMILLER. An *in vivo* and *in vitro* evaluation of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine: An inhibitor of human lymphoblast purine nucleoside phosphorylase. *Mol. Pharmacol.* 18: 287–295 (1980).

The synthetic nucleoside analog, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine (TCNR), is a competitive, reversible inhibitor of inosine phosphorolysis by human lymphoblast purine nucleoside phosphorylase. TCNR is not a substrate for the enzyme. The apparent K_i of TCNR is 5×10^{-6} M under conditions providing an apparent K_m of 2.5×10^{-5} M for inosine phosphorolysis. In both *in vivo* and *in vitro* assays TCNR is 7–10 times more potent than a previously described inhibitor of purine nucleoside phosphorylase, Formycin B. TCNR and Formycin B have growth inhibitory properties unrelated to inhibition of purine nucleoside phosphorylase. This additional effect of TCNR, inhibition of IMP dehydrogenase, is eliminated in studies using lymphoblast lines deficient in adenosine kinase, but this mutation has no effect on the growth inhibition produced by Formycin B. The primary effect of TCNR on purine nucleoside phosphorylase in intact cells is best demonstrated with a human lymphoblast line deficient in both adenosine kinase and hypoxanthine–guanine phosphoribosyltransferase which allows accumulation of inosine, guanosine, deoxyinosine, and deoxyguanosine in the medium. The accumulation of these nucleosides does not inhibit the growth of the human B lymphoblast.

INTRODUCTION

Two heritable disorders of the immune system are associated with deficiencies in enzymes involved in purine nucleotide catabolism and reutilization. A gross deficiency of adenosine deaminase (EC 3.5.4.4) is associated with dysfunction of T lymphocytes as well as varying degrees of B-lymphocyte dysfunction in different families (1). The deficiency of purine nucleoside phosphorylase (Puo phosphorylase,[1] purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) is associated with an isolated defect in all functions of T lymphocytes (2). In addition a marked decrease in ecto-5′-nucleotidase has been found in association with both hereditary (3) and acquired agammaglobulinemia (4) but whether or not the enzyme deficit represents the primary abnormal gene product or a secondary indication of arrested development of B cells remains to be proven. Considerable work has been done to elucidate the biochemical mechanism responsible for these immune dysfunctions (5–11). These experiments of nature provide models for development of highly specific inhibitors of these same enzymes as possible pharmacological agents that could provide highly specific suppression of the immune system.

The existence of several potent adenosine deaminase inhibitors [*erythro*-9-(2-hydroxy-3-nonyl)-adenine (12), coformycin, and 2′-deoxycoformycin (13, 14)] have afforded an opportunity to study the physiological role of adenosine deaminase in a variety of types of normal human cells and the molecular mechanisms by which the deficiency of adenosine deaminase may impair lymphocyte differentiation and function (14). Recent studies with these compounds have been directed toward development of suppressive agents of the immune system with

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[1] Abbreviations used: Puo phosphorylase, purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1); TCNR, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride; HPRT, hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8); Ado kinase, adenosine kinase (ATP:adenosine-5′-phosphotransferase, EC 2.7.1.20); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MH buffer, minimal Hepes buffer containing 135 mM NaCl, 32 mM Hepes, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.4% bovine serum albumin, pH 7.4.

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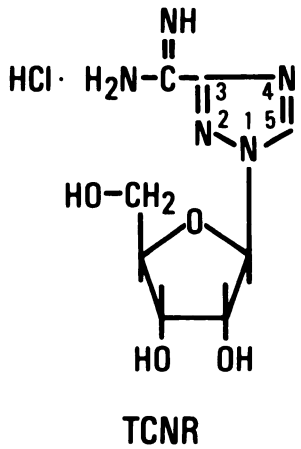
specific action on T cells (15). Numerous studies have described the use of these compounds to pharmacologically simulate under rigidly controlled laboratory conditions the adenosine deaminase-deficient state observed in the affected patient's cells. Some of these agents have also been used to potentiate the action of certain antiviral (16) and cancer chemotherapeutic agents (17). At the present time, only one inhibitor of Puo phosphorylase, Formycin B, has been described (18). The ratio of the K_i value of Formycin B over the K_m value for inosine (1×10^{-4} M/5×10^{-5} M = 2) indicates Formycin B is not a very effective inhibitor of Puo phosphorylase. Relatively high concentrations of Formycin B are therefore required to simulate the enzyme-deficient state of the immunodeficient patient's cells. Studies with cells from lymphoid or myeloid origin may be complicated by additional effects at high concentrations of Formycin B since marked leukopenia has been reported in dogs treated with doses of 50 mg/kg (18).

We have investigated a number of purine nucleoside analogs as inhibitors of cultured human lymphoblast Puo phosphorylase activity. One compound, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (TCNR) (19) (Fig. 1) proved to be superior to Formycin B as an inhibitor of Puo phosphorylase. The properties of this compound were investigated both with partially purified Puo phosphorylase and in intact cells using cultured human lymphoblasts. TCNR is bifunctional in normal cells inhibiting both Puo phosphorylase and, after metabolism which is dependent on adenosine kinase, IMP dehydrogenase.

MATERIALS AND METHODS

[^{14}C]Formate (59 mCi/mmol), [8-^{14}C]inosine (58 mCi/mmol), and [8-^{14}C]hypoxanthine (59 mCi/mmol) were purchased from Amersham/Searle. [8-^{14}C]Adenine (59 mCi/mmol) was purchased from New England Nuclear. α-Methylnaphthalene was purchased from Aldrich Chemical Company. Thin-layer cellulose chromatographic sheets were from Eastman Kodak (No. 13254) with fluorescent indicator No 6065. 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (TCNR) (19) and 8-aminoguanosine (20) were prepared by published procedures. Formycin B was obtained from Calbiochem. Calf spleen Puo phosphorylase was obtained from Sigma Chemical Company.



TCNR

FIG. 1. *Structure of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride*

Cell lines and medium. The lymphoblast lines used are of B-cell origin and have been described previously (21). The splenic-derived lymphoblast line WI-L2 (21) is used as a normal line. The derivatives of WI-L2 selected *in vitro* are: TGR-729CL6, a hypoxanthine phosphoribosyltransferase (HPRT; IMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.8)-deficient line; 107A, an adenosine kinase (Ado kinase; ATP:adenosine-5′-phosphotransferase, EC 2.7.1.20)-deficient line; and MTI-TG, a line deficient for both HPRT and Ado kinase. The cells were routinely cultured in RPMI 1640 medium containing 0.1% fetal calf serum (Irvine Scientific), 0.005% human transferrin (Sigma), and 0.4% bovine serum albumin (Sigma) as described (21). This medium was used in all studies of purine synthesis and excretion. For washing of lymphoblasts before preparation of cell lysates and for experiments with cells suspended in buffer alone, minimal Hepes buffer (MH buffer) was used and consisted of 135 mM sodium chloride, 32 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1.0 mM magnesium chloride, 0.1 mM calcium chloride, and 0.4% bovine serum albumin, pH 7.4 (22).

Purification of Puo phosphorylase. Growing cultures of lymphoblasts (200 ml at 1×10^6 cells/ml) were harvested, cooled to 4°C, and washed with MH buffer twice by centrifugation at 1500*g* for 10 min. The final cell pellet was resuspended to a density of 2×10^8 cells/ml in a buffer composed of 40 mM sodium Hepes, 32 mM sodium phosphate, 10 mM sodium ethylenediaminetetraacetic acid (EDTA), 0.6 mM, magnesium chloride, 0.5 mM β-mercaptoethanol, pH 7.4, and lysed by three cycles of freezing in liquid nitrogen and thawing at 37°C. The suspension was centrifuged at 3000*g* for 10 min and the pellet discarded and then at 100,000*g* for 60 min and the pellet again discarded. The high-speed supernatant materials were adjusted to 60% saturation by the slow addition of a saturated solution of ammonium sulfate. The pH was maintained at 7 by addition of ammonium hydroxide. After incubation at 4°C for 20 min, the suspension was centrifuged at 16,000*g* for 30 min and the precipitated material discarded. The supernatant solution was brought to 85% of saturation by three sequential dialyses over 24 h against 0.5 liter of 85% saturated ammonium sulfate and the supernatant material after centrifugation discarded. The precipitated material was resuspended in a minimum volume of water and dialyzed against 0.5 liter of 8 mM potassium phosphate, pH 7.4, changed three times in 13 h. The specific activity of Puo phosphorylase was 6 μmol/min/mg protein which represents a 10-fold purification over the value of the crude lysate. Protein was determined by the method of Lowry *et al.* (23).

Puo phosphorylase assays. Puo phosphorylase activity was measured by conversion of [8-^{14}C]inosine to [8-^{14}C]hypoxanthine as described by Thompson *et al.* (24). Unless stated otherwise, assays with cell extracts or partially purified enzyme were performed as follows. The assay mixtures were composed (in order of addition) of 25 μl of 0.4 M potassium phosphate buffer, pH 7.4, 5 μl of

Puo phosphorylase activity, 20 μl of inhibitor solution or water, and 5 μl of [8-^{14}C]inosine (sp act = 2 mCi/mmol, unless indicated otherwise). The assays were performed at 37°C, started by the addition of the isotope to prewarmed tubes containing all other components, and terminated by the addition of 10 μl of 8 M formic acid; and the tubes were maintained on ice. The assay mixtures were clarified by centrifugation at 5000*g* for 5 min. The product hypoxanthine was separated from inosine by chromatography of 20 μl reaction mixture with 5 μl of marker solution (0.5 mg/ml hypoxanthine, 1.0 mg/ml inosine) on cellulose sheets. The plates were developed to 10 cm above the origin with 1.6 M lithium chloride. The spots identified as inosine or hypoxanthine were cut out and counted in 3 ml of scintillation counting solution (22).

Puo phosphorylase activity of whole cells was determined in a similar manner except 100 μl of cell suspension was diluted 1:1 with 0.2 M sodium phosphate buffer, pH 7.4, containing inhibitor. After 2 min of incubation at 37°C, the assay was initiated by addition of 10 μl of [8-^{14}C]inosine. The assay was terminated by transferring 55 μl of incubation mixture to an ice-cold tube containing 10 μl of 8 M formic acid and treated as above.

The purine nucleoside analogs were evaluated as substrates for Puo phosphorylase by their donor activity in transribosylation assays (25, 26) which use the product of phosphorolysis, ribose 1-phosphate, as an intermediate in the synthesis of [8-^{14}C]inosine from [8-^{14}C]hypoxanthine as follows. Phosphorolysis:

nucleoside + phosphate ⇌ base + ribose 1-phosphate.

Synthesis:

ribose 1-phosphate + [8-^{14}C]hypoxanthine

⇌ phosphate + [8-^{14}C]inosine.

The reaction mixtures contained 17.5 μl of 0.2 M potassium phosphate buffer, pH 7.4, 7.5 μl of the partially purified Puo phosphorylase (sp act = 5.4 μmol/min/mg, 3 mg/ml), and 20 μl of the nucleoside analog. This mixture was incubated 20 min at 37°C and subsequently 5 μl of [8-^{14}C]hypoxanthine (12 mCi/ml) was added and the incubation continued for 40 min. The assay was terminated by the addition of 10 μl of 8 M formic acid and inosine synthesis determined after chromatography of 20 μl of assay mixture as described above.

Purine synthesis and excretion assays. Purine synthesis and excretion was determined by [^{14}C]formate incorporation as described by Herschfield and Seegmiller (27) and Willis *et al.* (21). For quantitation of the [^{14}C]formate incorporation, the purine bases were eluted from cation-exchange columns with 6 N HCl, triplicate samples were pooled, dried under vacuum, and reconstituted with water (approximate reconstitution volume = 100 μl water per 2.6 × 10^6 cells or 2.6 ml of culture medium). The purine bases were separated by chromatography of 10 μl of reconstituted materials with 2 μl of marker solution containing 0.5 mg/ml adenine, hypoxanthine, xanthine, and guanine on cellulose sheets. The plates were developed to 10 to 12 cm above the origin with a solvent system of butanol:acetonitrile:water (65:20:20). The distribution of formate-labeled materials was determined by fluorography as described (28) utilizing 0.4% 2,5-diphenyloxazole in α-methylnaphthalene. The areas containing radioactive materials were cut out and counted in 2 ml of scintillation counting solution.

High-pressure liquid chromatographic analysis. Samples for nucleotide analysis and nucleoside/base analysis of cells and medium, respectively, were prepared by centrifugation of cultures at 1500*g* for 5 min. The medium was transferred to an iced tube containing 0.1 vol of 4.4 N perchloric acid. The cell pellet was resuspended in cold 0.4 N perchloric acid (100 μl/2 × 10^6 cells). After 5 min, the precipitated materials were removed by centrifugation and the resulting supernatant materials neutralized with alamine/Freon as described by Khym (29). The samples were stored at −20°C until analysis. Nucleotide analysis was performed on 100 μl of sample by chromatography at ambient temperature on a Partisil PXS/10/25 SAX column (Whatman) developed by a gradient formed with buffer A (10 mM potassium phosphate, pH 3.77) and buffer B (0.25 M potassium phosphate–0.5 M potassium chloride, pH 3.45) with a combined flow rate of 1.5 ml/min as follows: 6 min of buffer A; 6 min of linear increase to 30% buffer B; 7 min of linear increase from 30 to 100% buffer B; 24 min of 100% buffer B. An 8-μl flow cell was used and the absorbance at 254 nm was monitored at 0.04 *A* full scale. Peak areas were determined with a Spectra Physics I computing integrator. The nucleotides were quantitated by comparison of areas to areas of standards. Nucleoside/base analysis was performed on 100-μl samples of medium by chromatography at ambient temperature on a Bondapak C18 P/N 27324 reverse-phase column (Waters Associates) developed with a gradient formed from buffer A (5 mM potassim phosphate, pH 3.34) and buffer B (60% acetonitrile in water) at a combined flow rate of 1.5 ml per minute. A 15-min-long linear gradient program from 0 to 15% was used. Absorbance at 254 and 280 nm was monitored as described above.

RESULTS

Properties of the lymphoblast Puo phosphorylase. The cultured human lymphoblast Puo phosphorylase exhibits substrate activation suggestive of positive homotrophic cooperative effects with inosine as the substrate; this effect is compared to results with a commercial preparation of the enzyme from calf spleen in Fig. 2. With low concentrations of inosine, <35 μM, the lymphoblast enzyme exhibits an apparent K_m for inosine of 25 μM. This value is consistent with the K_m value determined for the calf spleen enzyme over all inosine concentrations studies, 5–1500 μM. With inosine concentrations above 35 μM, higher apparent K_m values approaching 200 μM are observed.

Inhibitory and substrate properties of TCNR. Figures 3 and 4 present the results of kinetic analyses of TCNR and the determination of the K_i values through the use of double-reciprocal plots with subsequent replotting of the slopes versus the inhibitor concentration. At low concentrations of inosine, <35 μM, which provide an apparent K_m of 25 μM, the inhibition of TCNR appeared

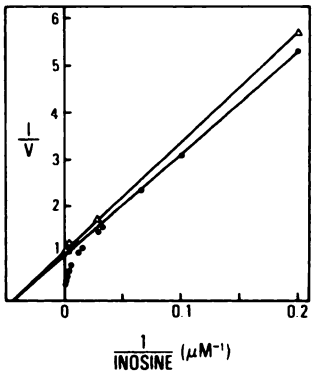


FIG. 2. *Double-reciprocal plots of initial velocity and concentration of inosine with the human lymphoblast* (●) *and bovine spleen* (△) *Puo phosphorylase*

The initial velocities (μM/min) were graphically determined from plots of inosine conversion to hypoxanthine versus time with each inosine concentration. The specific activities of [^{14}C]inosine were 58 mCi/mmol over the range 5 to 35 μM and 4 mCi/mmol over the range 67 to 1500 μM.

to be competitive and the K_i value of TCNR was estimated to be as low as 5 μM (Fig. 3). However, the replot of the slope versus TCNR concentration was not linear over the range of TCNR concentrations studied but characteristic of hyperbolic competitive inhibition. This effect could be due to activation of the enzyme by either the substrate or the inhibitor or the combination. In a parallel study, a K_i value of 4 μM was determined for TCNR with the calf spleen enzyme and the replot of the slope versus inhibitor concentration was linear. The use of higher concentrations of inosine with the lymphoblast enzyme (Fig. 4) provides an apparent K_m of 200 μM and the replot of the slope versus TCNR concentration appeared to be linear, suggesting the enzyme may be approaching full activation. An apparent K_i value for TCNR of 50 μM was determined.

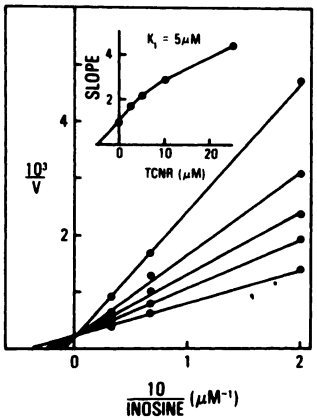


FIG. 3. *Determination of the apparent K, value for TCNR with low concentrations of inosine as substrate for the lymphoblast Puo phosphorylase*

The initial concentrations of [^{14}C]inosine (sp act 58 mCi/mmol) were 5, 15, and 30 μM. Utilizing a Lineweaver-Burk plot of 1/velocity (cpm in hypoxanthine/3 min per 20 μl of assay mixture) values versus 1/initial inosine concentration, a slope and apparent K_m value were determined for each TCNR concentration. The apparent K_m value in the absence of TCNR was 25 μM. A replot (inset) of slope value versus TCNR concentration gave the inhibition constant for TCNR.

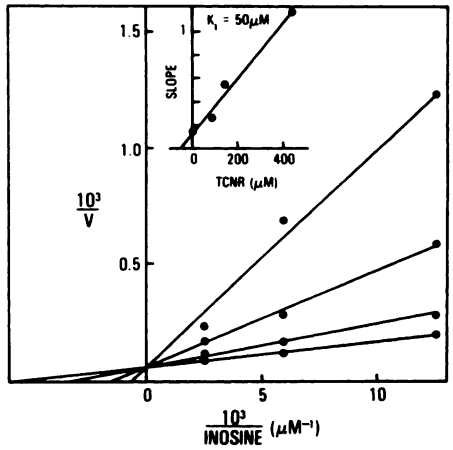


FIG. 4. *Determination of the K, value for TCNR with high concentrations of inosine as substrate for the lymphoblast Puo phosphorylase*

The initial concentrations of inosine (sp act 2 mCi/mmol) were 80, 170, and 250 μM. Utilizing a Lineweaver-Burk plot of 1/velocity (cpm in hypoxanthine/15 min per 20 μl of assay mix) values versus 1/initial inosine concentration, a slope and apparent K_m value were determined for each TCNR concentration. The apparent K_m value in the absence of TCNR was 200 μM. A replot (inset) of slope values versus TCNR concentrations gave the inhibition constant for TCNR.

proaching full activation. An apparent K_i value for TCNR of 50 μM was determined.

The ratios of the K_i values of TCNR over the K_m values for inosine of 0.2 (Fig. 3) and 0.3 (Fig. 4) suggest TCNR is a better inhibitor of inosine phosphorolysis than Formycin B whose ratio is 2 (18). The inhibitory properties of Formycin B were compared to those of TCNR (Fig. 5). TCNR is aproximately 10 times more potent as an inhibitor of inosine phosphorolysis.

Irreversible effects of either TCNR or Formycin were not observed when Puo phosphorylase was exposed to 2 mM inhibitor for 2 h and the incubation mixtures were subsequently diluted 10-fold for analysis of nucleoside phosphorylase activity.

TCNR was evaluted as a ribose 1-phosphate donor in assays measuring transribosylation (25, 26) with hypo-

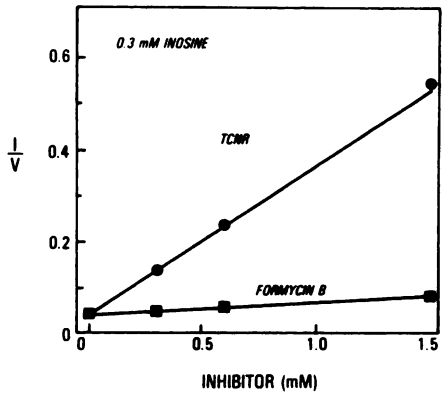


FIG. 5. *The inhibition of Puo phosphorylase by TCNR* (●) *and Formycin B* (■)

Puo phosphorylasse assays were performed as described under Materials and Methods with partially purified lymphoblast enzyme. The assays were 15 min in length. V = [8-^{14}C]hypoxanthine/([8-^{14}C]inosine + [8-^{14}C]hypoxanthine) × 100. The data indicated are the averages of triplicate determinations.

xanthine (Table 1) and nucleoside-dependent adenine conversion to nucleotide as described by Skaper *et al.* (30); essentially no phosphorolysis of this nucleoside was detected. 8-Aminoguanosine was included since this nucleoside has the lowest apparent K_i values, 1 and 4 μM, of all inhibitors studied as described for TCNR in Fig. 3 and 4, respectively. 8-Aminoguanosine is a substrate for Puo phosphorylase but is poor relative to inosine and guanosine (26).

As shown in Fig. 6, when included in suspensions of intact lymphoblasts TCNR is as potent an inhibitor of [8-^{14}C]inosine phosphorolysis as is unlabeled inosine. This result suggests that the lymphoblast is permeable to the TCNR but does not eliminate the possibility that TCNR may also be an inhibitor of inosine transport into the cell.

Toxicity to normal and Ado kinase-deficient lymphoblasts. Both Formycin B and TCNR are toxic to cultures of the lymphoblast line WI-L2 (Table 2). Formycin B at 5 μM inhibits the growth rate of WI-L2 cultures to 33% of control values and at concentrations of 15 μM complete growth arrest is observed. TCNR at 100 μM inhibits the growth rate of WI-L2 to 50% of control values and at concentrations of 500 μM complete growth arrest is observed. 8-Aminoguanosine was not toxic at 500 μM.

The toxicity of TCNR and Formycin B was evaluated in cultures of WI-L2 derivatives deficient in either Ado kinase, HPRT, or both Ado kinase and HPRT (Table 2). Ado kinase-deficient lines were resistant to growth arrest by 500 μM TCNR. However, none of the enzymatic deficiencies resulted in resistance to Formycin B. We previously have shown that ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a compound structurally related to TCNR, is a potent inhibitor of IMP dehydrogenase and subsequently inhibits the growth of cultures when phosphorylated by the Ado kinase activity of the cells (21). These effects of ribavirin are not observed with Ado kinase-deficient lymphoblasts. Evidently, TCNR, like ribavirin, must be metabolized via Ado kinase activity to inhibit growth; the mechanism of growth inhibition is evaluated below.

Effects on purine nucleotide content. The mechanism of growth inhibition by purine-related antimetabolites was explored by analysis of the purine nucleotide content of cells after exposure to the drugs. In previous studies, the inhibition of the IMP dehydrogenase activity characteristically caused a diminished guanylate nucleotide pool (21, 31, 32); the inhibition (33) or deficiency (34) of adenylosuccinate synthetase activity, a diminished adenylate pool. As shown in Table 3, the presence of TCNR in WI-L2 cultures causes the GTP and GMP content of the lymphoblast to decrease to approximately 10 and 20% of control values, respectively. The IMP

TABLE 1

Substrate activity of purine nucleoside analogs

Nucleoside	Ribosyl donor activity	
	μM[a]	%[b]
Inosine	15.8	100
Guanosine	14.3	91
2′-Deoxyinosine	4.4	28
2′-Deoxyguanosine	4.1	26
8-Aminoguanosine	2.9	18
Formycin B	0.4	3
TCNR	0.1	1

[a] The concentration of [8-^{14}C]inosine synthesized from 200 μM [8-^{14}C]hypoxanthine and the ribose 1-phosphate donated by 222 μM purine nucleoside analog. Each value is the average of triplicate determinations. Values of 0.4 μM or less are not considered significant.

[b] Relative concentration of [8-^{14}C]inosine formed from [8-^{14}C]hypoxanthine and the ribose 1-phosphate donated from phosphorolysis of the nucleoside analog (concentration with inosine = 100%).

TABLE 2

The toxicity of TCNR and Formycin B to cultures of WI-L2 and enzyme-deficient derivatives

Cultures were initiated at 0.5 × 10^5 cells per milliliter in medium containing the indicated concentrations of inhibitors. Cultures were counted daily with a Coulter ZB I counter. Growth rate is expressed as generations per hour based on the growth increment observed between 48 and 72 h of culture.

Deficiency	Inhibitor	Concentration (μM)	Growth rate (h^{-1})
None	None	—	0.042
	TCNR	5	0.041
		20	0.032
		100	0.019
		500	−0.020
	Formycin B	5	0.013
		15	−0.019
		50	−0.056
		500	−0.056
Ado kinase	None	—	0.045
	TCNR	500	0.042
	Formycin B	500	−0.063
HPRT	None	—	0.048
	TCNR	500	−0.027
	Formycin B	500	−0.058
Ado kinase, HPRT	None	—	0.048
	TCNR	500	0.044
	Formycin B	500	−0.060

TABLE 3

The effect of TCNR on the purine nucleotide content of WI-L2 and the Ado kinase-deficient derivative

Cultures of WI-L2 and the Ado kinase-deficient line, 107 A, were initiated at 0.5 × 10^6 cells per milliliter in medium containing 500 μM TCNR. The cultures were harvested after 24 h, acid-soluble extracts of cells were prepared, and nucleotide analysis was performed by anion-exchange, high-pressure liquid chromatography as described under Materials and Methods.

Culture		Nucleotide content (nmol/10^6 cells)					
Line	TCNR	IMP	Adenylates[a]		Guanylates		
			ADP	ATP	GMP	GDP	GTP
WI-L2	−	0.09	0.85	3.49	0.15	0.49	1.32
	+	0.61	0.79	3.53	0.03	0.48	0.14
107A	−	0.10	0.73	3.12	0.17	0.33	1.14
	+	0.16	0.77	3.10	0.16	0.29	1.07

[a] AMP was not separable from NAD in this series of analysis.

content is increased sixfold and the adenylate content and distribution are unaffected. In addition, the UTP content is elevated approximately twofold and the UDP sugar content approximately three-fold (data not shown). These data are consistent with TCNR inhibiting IMP dehydrogenase activity (31, 32). On the other hand, the purine nucleotide content of the Ado kinase-deficient lymphoblast exposed to TCNR is not significantly different from the nucleotide contents of control cultures. The purine nucleotide content of cells incubated with Formycin B also is not different from that of control cultures although the growth of the lymphoblast cultures is restricted by the drug.

Effects on purine synthesis and excretion. TCNR stimulates both purine synthesis *de novo* and purine excretion by WI-L2 cells (Table 4). After 1 h incubation of the cultures with 100 or 500 μM TCNR purine synthesis is elevated 20 and 50%, respectively, and purine excretion increases from 4% to 24% and 47%, respectively. Guanylate synthesis is relatively unaffeted by 100 μM TCNR but is inhibited 44% with 500 μM TCNR. The increased purine synthesis observed when WI-L2 cells are exposed to 100 μM TCNR is not evident with the HPRT-deficient cells. However, the effect of 500 μM TCNR on the HPRT-deficient line is similar to the effect of WI-L2; purine synthesis and excretion are increased by 42 and 65%, respectively, and guanylate synthesis is inhibited 53%. With the incubation times used in these studies, TCNR has two effects which are dependent on its concentration With 100 μM TCNR, purine excretion and correspondingly total purine synthesis by the normal line are elevated without inhibition of guanylate synthesis; HPRT-deficient lines are unaffected. Therefore, 100 μM TCNR increases the rate of purine excretion to values comparable to those of the HPRT-deficient cells. With 500 μM TCNR, total purine synthesis and excretion are elevated and guanylate synthesis is inhibited in both the normal and HPRT-deficient lines; this second effect is due to inhibition of IMP dehydrogenase.

The response of the Ado kinase-deficient lymphoblast to the presence of 100 μM TCNR resembles that of the WI-L2 line. Purine excretion is increased from 4 to 24% and total purine synthesis correspondingly increases. However, with 500 μM TCNR, the second effect of TCNR, i.e., a further increase of purine synthesis and excretion concomitant with an inhibition of guanylate synthesis, is not observed. This result provides further evidence that the second effect of TCNR is mediated by Ado kinase and involves conversion of the TCNR nucleoside to its nucleotide. This TCNR nucleotide or a derivative thereof then inhibits the IMP dehydrogenase-dependent conversion of IMP to GMP. With the short exposure times used, i.e., less than 3 h, the primary effect of TCNR is the inhibition of purine nucleoside phosphorylase activity. This effect is indistinguishable in [^{14}C]formate incorporation assays from a genetic deficiency of HPRT. The line deficient for both Ado kinase and HPRT as expected is not affected by exposure to either 100 or 500 μM TCNR.

In similar experiments, Formycin B inhibited *de novo* purine synthesis of WI-L2 cells. The inhibition was pro-

TABLE 4

The effect of TCNR on purine synthesis, base distribution, and excretion by WI-L2 and the enzyme-deficient derivatives

Cultures (1.0 ml) were initiated at 1 × 10^6 cells per milliliter and allowed to equilibrate at 37°C for 30 min. TCNR (0.05 ml) was added and cultures were incubated 60 min. [^{14}C]Formate (0.010 ml, 1 mCi/ml) was then added and the cultures were incubated for 90 min before the determination of formate incorporation into purines.

Culture		[^{14}C]Formate incorporation into purines[a]									
		Cells				Medium				Total	
		Distribution			Rate	Distribution			Rate		
Deficiency	TCNR (μM)	A	HX	G		A	HX	G		Rate[b]	Excreted[c] (%)
None	0	10.6	0.7	10.8	22.1	0.1	0.6	0.3	1.0	23.1	4
	100	11.0	0.6	9.1	20.7	0.1	6.1	0.5	6.7	27.4 (1.19)	24
	500	10.6	1.9	6.1	18.6	0.2	14.7	1.4	16.3	34.9 (1.51)	47
HPRT	0	7.4	0.8	7.7	15.9	0.1	8.4	0.6	9.1	25.0	36
	100	7.4	0.8	7.7	15.9	0.1	8.6	0.7	9.4	25.3 (1.01)	37
	500	7.3	1.6	3.6	12.5	0.2	22.0	0.9	23.1	35.6 (1.42)	65
Ado kinase	0	11.3	0.6	7.6	19.5	0.2	1.0	0.2	1.4	20.9	7
	100	10.9	0.8	8.2	19.9	0.2	5.5	0.6	6.3	26.2 (1.25)	24
	500	10.0	1.0	8.4	19.4	0.1	5.4	1.0	6.5	25.9 (1.24)	25
Ado kinase, HPRT	0	9.1	0.5	6.6	16.2	0.3	6.4	0.5	7.2	23.4	31
	100	9.0	0.7	6.8	16.5	0.2	5.6	0.6	6.4	22.9 (0.98)	28
	500	8.5	0.8	7.6	16.9	0.2	5.6	0.9	6.7	23.6 (1.01)	28

[a] [^{14}C]Formate incorporated into adenine (A)-, hypoxanthine (HX)-, and guanine (G)-containing compounds: cpm × 10^{-3} per 0.2 × 10^6 cells per 1.5 h.

[b] The sum of cell and medium formate incorporation rates. Numbers in parentheses refer to the total rate in the presence of TCNR divided by the total rate in the absence of TCNR.

[c] Formate incorporation into purine components found in the culture medium divided by total formate incorporation into purines by the culture × 100.

gressive and the proportion of newly synthesized purine appearing in the adenylates and guanylates was not remarkably altered. In contrast, purine synthesis by the HPRT-deficient lines are relatively resistant to Formycin B for 3 to 4 h of exposure, even though this compound restricted growth of HPRT-deficient lymphoblasts to the same extent as observed with WI-L2. These discordant results suggest additional mechanisms for Formycin B action not previously recognized.

Distribution of purines accumulated in the culture medium. Figure 7 provides a profile of the excretion of hypoxanthine and inosine into the culture medium by the HPRT-Ado kinase-deficient lymphoblast. The HPRT-deficient lymphoblasts accumulate in the medium 15 to 30% of the total purine synthesized per generation of culture. The rate of purine excretion is approximately 2.5 nmol/h per 10^6 cells. The excreted purine is primarily hypoxanthine, but a small quantity of inosine is observed. In the presence of Formycin B approximately two to three times as much inosine accumulates during the initial period of incubation of the cultures; however, the major component of the purine excreted remains hypoxanthine. This observation indicates Formycin B at a very toxic concentration is only marginally effective in the inhibition of Puo phosphorylase activity of the culture. A steady-state concentration of 1 to 1.5 μM inosine appears to be maintained with 500 μM Formycin B. The additional toxicity associated with Formycin B is reflected in the diminished rate of purine excretion by the cultures after 4 h of exposure to the drug. In the presence of 500 μM TCNR a similar amount of total purine is excreted into the medium but during the initial 2 to 3 h of culture the principal purine accumulating in the medium is inosine. With continued culture, the inosine content of the culture medium attains a steady-state concentration of 9 to 10 μM after which hypoxantine continues to increase in parallel with the hypoxanthine accumulation observed in the control culture. In addition guanosine, 2′-deoxyinosine, and 2′-deoxyguanosine also accumulate in the presence of 500 μM TCNR; the steady-state concentrations observed after 24

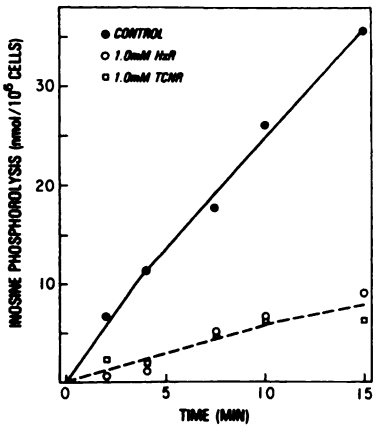


FIG. 6. *The evaluation of TCNR as an inhibitor of inosine phosphorolysis by suspensions of intact lymphoblasts*

Suspensions of the HPRT-deficient lypmphoblast TG-729CL6 were incubated as described under Materials and Methods for the times indicated with 0.2 mM [8-^{14}C]inosine alone (●) or including 1 mM unlabeled inosine (□) or 1 mM TCNR (○).

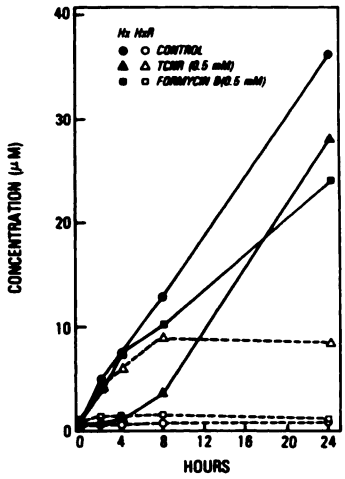


FIG. 7. *The effect of TCNR and Formycin B on the distribution of hypoxanthine-containing compounds excreted into the culture medium*

Cultures of the Ado kinase-HPRT-deficient lymphoblast MTI-TG were initiated at 0.5×10^6 cells/ml in medium containing 0.5 mM TCNR (triangles), 0.5 mM Formycin B (squares), or no drug (circles). At the times indicated, 2-ml samples of the cultures were removed and the medium was processed for high-pressure liquid chromatographic determination of the nucleoside and base content. Closed symbols, hypoxanthine; open symbols, inosine.

h were 1.2, 0.7, and 0.6 μM, respectively. In these *in vivo* studies TCNR is six to seven times more effective than Formycin B as an inhibitor of Puo phosphorylase, but as evidenced by the time-dependent hypoxanthine accumulation, TCNR is not as potent an inhibitor as may be desired for some studies or to mimic the enzyme-deficient state observed in lymphoblasts of patients affected with the deficiency of Puo phosphorylase.

DISCUSSION

These studies have shown TCNR to be superior to Formycin B as a competitive inhibitor of the Puo phosphorylase activity of cultured human lymphoblasts. TCNR like Formycin B (18) does not appear to be phosphorylised by the enzyme. Both TCNR and Formycin B have additional effects. TCNR inhibits IMP dehydrogenase; this inhibition occurs after an adenosine kinase-dependent conversion of TCNR to a nucleotide. In this respect, TCNR is similar to the structural analog ribavirin (21). Whether or not other enzymatic conversions of TCNR occur or whether or not the nucleotide derivative of TCNR must undergo further metabolism to be active as an inhibitor of IMP dehydrogenase remains to be determined.

Formycin B appears to have multiple effects on the metabolism of the human lymphoblast which have not been previously recognized (18). These secondary targets have not been clearly defined. Short periods of exposure of normal lymphoblasts to Formycin B inhibit purine synthesis. Longer periods of exposure were required for similar effects to be observed in HPRT-deficient lymphoblasts. The toxicity of Formycin B is not consistent with inhibition of Puo phosphorylase and was apparent

and similar in HPRT-deficient, Ado kinase-deficient, and normal lines.

The effects of TCNR specific to Puo phosphorylase were investigated in two ways. First, in short-term studies of 2- to 4-h duration, 100 μM TCNR primarily inhibits Puo phosphorylase. Normal cells exposed to this concentration of drug are indistinguishable in assays of purine synthesis and excretion from cells which are genetically deficient for HPRT or Puo phosphorylase. The newly formed purines entering into the "IMP futile cycle" (21) are lost from the cell as hypoxanthine in the case of HPRT deficiency and as inosine in case of genetic or TCNR-induced Puo phosphorylase deficiency. With assays of longer duration or with concentrations of TCNR above 100 μM, the adenosine kinase-mediated inhibition of IMP dehydrogenase develops. Guanylate synthesis is inhibited and a further increase in excretion of newly formed purine occurs. The stimulation of purine synthesis when IMP dehydrogenase activity is inhibited is presumably due to diminished guanylate feedback control on glutamine amidotransferase (21, 35, 36). Since cells deficient in Ado kinase fail to show these effects, the second approach in the investigation of effects of TCNR specific to Puo phosphorylase is to use lymphoblasts with this metabolic defect. Higher concentrations of TCNR can be used and the drug can be maintained in the culture system for long periods without adverse effects. In studies with cells genetically deficient for Ado kinase, we have shown the accumulation in the culture medium of purine nucleosides to steady-state levels which markedly exceed steady-state levels observed without TCNR present or with equimolar concentrations of Formycin B present. The degree of inhibition of Puo phosphorylase achieved with TCNR in these studies, while more pronounced than with Formycin B, does not affect the growth of lymphoblasts. Caution should be exercised, therefore, in the interpretation of growth inhibiton results with Formycin B. However, these studies with TCNR also show continued accumulation of large amounts of hypoxanthine, which is indicative of an incomplete block of Puo phosphorylase. Evidently, an inhibitor with higher affinity for Puo phosphorylase is required to fully mimic the enzyme-deficient state of cells from a patient with genetic deficiency of Puo phosphorylase (7, 8)

As a model compound of a purine nucleoside phosphorylase inhibitor, TCNR may provide the prototype for future development of a more specific inhibitor with higher affinity for Puo phosphorylase. We are presently exploring two positional substitutions of TCNR. These are the α- and β-2′-deoxyribose analogs and a 5-amino substitution of the triazole ring. The 2′-deoxy analogs were chosen since then may not be metabolized to a nucleotide or if metabolized to a nucleotide may not be effective inhibitors of IMP dehydrogenase. The 5-amino substitution of TCNR was chosen since an amino group at this position may act like the amino group at the 8 position of adenosine or guanosine. This substitution significantly decreases the K_i values of the nucleoside for Puo phosphorylase of *Escherichia coli* (25), the human erythrocyte (26), and, as shown in this study, the human lymphoblast.

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