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Action of 2-β-D-Ribofuranosylthiazole-4-carboxamide (Tiazofurin) in Chinese Hamster Ovary and Variant Cell Lines

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SUMMARY

The metabolism and inhibitory activity of 2-β-D-ribofuranosylthiazole-4-carboxamide (tiazofurin, RTC, NSC 286193) was studied in Chinese hamster ovary (CHO) cells and a series of mutants derived from them. Isotope incorporation experiments indicate dramatic inhibition of DNA and RNA synthesis by tiazofurin but little or no effect on protein synthesis. In CHO cells, tiazofurin is more toxic than ribavirin and also demonstrates a stronger inhibition of guanine nucleotide synthesis. Tiazofurin is phosphorylated by crude extracts of CHO cells. Variant cell lines lacking adenosine kinase (EC 2.7.1.20) or deoxycytidine kinase (EC 2.7.1.74) show no resistance to tiazofurin, suggesting that these enzymes are not responsible for the phosphorylation of tiazofurin. The inhibitory activity of this agent can be reversed by exogenously supplied guanine, suggesting that the inhibition is due, at least in part, to guanylate deficiency.

INTRODUCTION

The recent synthesis of tiazofurin[3] (RTC, NSC 286193) (1), an analogue of the antiviral agent ribavirin (2), has provided an interesting and potentially useful antitumor agent. This compound is unusual in having demonstrated remarkable activity against Lewis lung carcinoma (3). It has been found in various systems (1, 4) to depress guanine nucleotide synthesis markedly as a result of IMP dehydrogenase (IMP:NAD oxidoreductase, EC 1.2.1.14) inhibition. Formation of the phosphorylated form of tiazofurin has been demonstrated with crude cell extracts (5); however, the potent IMP dehydrogenase inhibition has been ascribed to the formation of an analogue of NAD in which tiazofurin has replaced the nicotinamide moiety (5, 6). This could presumably occur through the action of ATP:NMN adenylytransferase (EC 2.7.7.1), which has been shown to accept tiazofurin-5′-monophosphate as a substrate in place of nicotinamide mononucleotide (6). This communication addresses the action of tiazofurin in CHO cells and drug-resistant cell lines derived from them and compares the action of this agent with those of the structurally related compounds ribavirin and pyrazofurin (Fig. 1).

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[3] The abbreviations used are: tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide; CHO cell, Chinese hamster ovary cell; ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; pyrazofurin, 3-β-D-ribofuranosyl-4-hydroxypyrazole-5-carboxamide; ara-C, 1-β-D-arabinofuranosylcytosine; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; HGPRT, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); IMP dehydrogenase, IMP:NAD oxidoreductase (EC 1.2.1.14).

MATERIALS AND METHODS

Chemicals. Tiazofurin (RTC, riboxamide), and its 5′-deoxy derivative were prepared as previously described (1). Ribavirin was prepared by the procedure of Witkowski *et al* (2). Pyrazofurin was purchased from Calbiochem Corporation (La Jolla, Calif.). All other reagents were purchased from appropriate commercial sources.

Cells and medium. CHO cells were carried in monolayer culture using McCoy's 5A growth medium supplemented with 10% fetal calf serum. Dialyzed fetal calf serum was used in all experiments relating to drug inhibition and utilization to avoid competition by natural serum components. This serum was routinely prepared by dialyzing whole fetal calf serum against 10 volumes of PBS for 5 days at 4° with four changes of PBS. The serum was sterilized by passage through millipore filters having a pore size of 0.45 μm. Cell counts of suspensions obtained by trypsinization were taken with the aid of an electronic particle counter (Model ZBI, Coulter Electronics). The mutant cell line, Rb^R-1, was derived from the CHO line by conventional methods of mutagenesis, was selected with ribavirin, is deficient in adenosine kinase activity, and is described elsewhere (7). Line aC^R-7 was similarly derived, selected with ara-C, is deoxycytidine kinase-deficient, and is also described elsewhere (8). Lines RO^R-1 and RO^R-2 (tiazofurin-resistant) were selected with 100 μM tiazofurin. Line TG^R-3 was selected with 100 μM 6-thioguanine and is deficient in HGPRT.

Determination of minimal inhibitory drug concentra-

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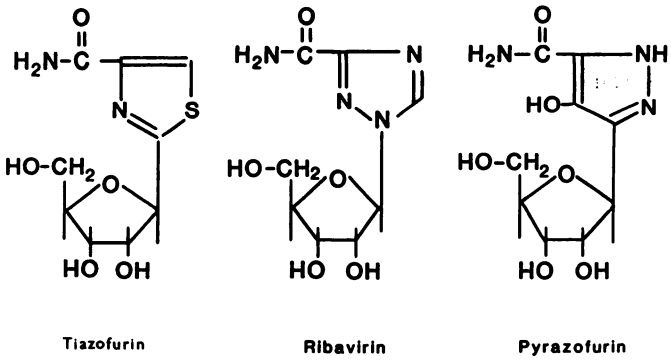

FIG. 1. *Structures of the related compounds tiazofurin, ribavirin, and pyrazofurin*

tions by inhibition of clone formation. Logarithmically growing CHO cells were trypsinized, diluted with medium containing dialyzed fetal calf serum, dispensed into a series of 35-mm plastic Petri dishes (approximately 200 cells/dish), and allowed to form clones in a broad range of drug concentrations: 0.5, 1, 5, 10, 50, 100, 500, and 1000 μM. After 7–8 days' incubation in a humidified CO_2 incubator at 37.5° the media were poured off and the clones fixed with formaldehyde followed by staining with crystal violet. The minimal inhibitory drug concentration is the lowest tested concentration of drug that results in the formation of clones containing fewer than 50 cells.

Determination of plating efficiencies. Cells were dispensed into sterile 20-ml glass vials, 5×10^4 cells/vial, in medium containing dialyzed serum. After a 1.5-hr incubation to allow attachment the medium was aspirated and replaced with fresh medium containing the indicated additions (in duplicate). The vials were returned to the incubator and at appropriate intervals vials were removed, the medium was aspirated, and the cell monolayer was washed twice with PBS. The cells were detached with trypsin, disaggregated by repeated pipetting, and appropriately diluted for plating in 35-mm plastic Petri dishes. Growth of clones was allowed for 8 days, after which the clones were stained as described above and counted.

Phosphorylation of tiazofurin by crude cell extracts. Procedures employed for preparation of the extract and incubation with tiazofurin are essentially as described elsewhere (5). Cells were harvested from roller bottles during exponential growth, suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4) (2×10^8 cells/10 ml) containing 1 mM dithiothreitol, and sonicated. The sonicate was centrifuged at 10,000 × *g* for 30 min and the supernatant solution was treated with ammonium sulfate to 60% saturation. The precipitated proteins were dissolved in 0.05 M Tris-HCl (pH 7.4) containing 1 mM dithiothreitol and dialyzed overnight against the same buffer at 4°.

Dialyzed extract (350–500 μg of protein) was incubated with 1 mM tiazofurin, 8 mM ATP, 5 mM $MgCl_2$, and 0.05 M Tris-HCl (pH 7.4) in a final volume of 0.1 ml for 1 hr at 37°. The reaction was stopped by the addition of perchloric acid (0.4 N) and centrifuged. After neutralization of the supernatant with KOH and removal of the precipitate by centrifugation, the extract was analyzed by HPLC. HPLC was performed with a Waters Associates ALC 204 high-pressure liquid chromatograph equipped with two Model 6000A pumps, U6K injection system, Model 660 A gradient programmer, and a column of Partisil 10 SAX anion exchange resin (Whatman, Inc., Clifton, N. J.). Elution was with a gradient of ammonium phosphate [0.005 M (pH 2.8) and 0.75 M (pH 3.7), 0–30% in 30 min]. The compounds were detected with a Model 440 detector and quantitated with a Data Module.

Measurement of cellular uptake of isotopes and incorporation into perchloric acid-insoluble materials. Logarithmically growing CHO cells were trypsinized and dispensed into sterile 20-ml glass vials, 1×10^5 cells/vial in 2 ml of medium, and incubated for 16–18 hr in a humidified CO_2 incubator at 37°. The medium in each vial was then removed and replaced with 1 ml of fresh medium containing dialyzed serum, isotopically labeled precursor (2 μCi/ml), and other desired additions. Precursors employed were [*methyl*-^{3}H]thymidine (15 Ci/mmole), [5-^{3}H]uridine (26.7 Ci/mmole), and [4,5-^{3}H]leucine (54 Ci/mmole). The vials were returned to the incubator and after 4 hr vials were removed and the media were quickly aspirated. After washing the cells twice with 10 ml of cold PBS, 1 ml of 0.4 N perchloric acid was added to each vial. The vials were allowed to stand in an ice bath for at least 2 hr, after which the perchloric acid solutions were removed. To determine

TABLE 1

Growth inhibition of CHO and variant cell lines

Procedures were as described in text. Determination of enzyme activities were as described elsewhere (8). Specific activities are expressed as nanomoles of product formed per minute per milligram of protein.

Cell line	Enzyme[a] specific activities		Minimal inhibitory concentration				
	AK	dCK	Ribavirin	Pyrazofurin	Ara-C	Tiazofurin	5′-Deoxy-tiazofurin
					μM		
CHO	1.12	0.023	50	50	0.5	5.0	500
Rb^R-1	<0.01	0.029	500	500	0.5	5.0	ND[b]
aC^R-7	1.29	0.005	50	50	50.0	5.0	ND
RO^R-1	1.90	0.018	10	50	0.5	500.0	ND
RO^R-2	1.20	0.019	10	50	0.5	500.0	ND

[a] AK, adenosine kinase (EC 2.7.1.20); dCK, deoxycytidine kinase (EC 1.2.1.74).

[b] ND, Not determined.

TABLE 2

Phosphorylation of tiazofurin by extracts of CHO and mutant cell lines

The procedure is described under Materials and Methods.

Cell line	Tiazofurin monophosphate formed[a]
CHO	2.08
RO^R-1	1.73
RO^R-2	0.89
Rb^R-1	1.34

[a] Values are expressed as nanomoles of tiazofurin monophosphate formed per minute per milligram of protein.

the perchloric acid-insoluble radioactivity, the residues in the vials were washed twice with 0.4 N perchloric acid and counted with 10 ml of counting solution.

The method of Hershfield and Seegmiller (9) was used to measure the effects of tiazofurin and related compounds on *de novo* purine nucleotide synthesis. Briefly stated, cells were preincubated with drug followed by the addition of [^{14}C]formate and additional incubation. The cell monolayer was washed, 0.4 N perchloric acid was added, and the nucleotides were hydrolyzed by heating at 100°. After passage through a small AG-50 column and elution of purine bases with 6 N HCl, the fractions were concentrated by evaporation and the amount of ^{14}C in each purine base was determined by thin-layer chromatography.

Analysis of cellular nucleotide pools. Cells were dispensed into 250-ml tissue culture flasks (5×10^6 cells/flask) in 20 ml of medium and incubated for 18 hr in a humidified CO_2 incubator at 37°. The media were then aspirated and replaced with 10 ml of medium containing dialyzed serum and the desired additions. A duplicate flask was used to determine the number of cells present. After 4–6 hr of incubation the media were aspirated and the monolayers were quickly washed with cold PBS followed by extraction of nucleotide pools with cold 0.4 N perchloric acid. After neutralization with KOH the nucleotide pools were fractionated using a Waters Associates (Milford, Mass.) ALC 204 high-pressure liquid chromatograph equipped with two Model 6000A pumps, Model 660 gradient programmer, and a column of Partisil-10 SAX anion exchange resin (25 cm × 4.6 mm; Whatman Inc.). Cell samples (2.5×10^6 cell equivalents) were injected by means of the U6K-LC injection system and eluted with a linear gradient [40 min; from 0.150 M $NH_4H_2PO_4$ (pH 2.8) to 0.750 M $NH_4H_2PO_4$ (pH 3.7)] (10) at a flow rate of 2 ml/min. One-milliliter fractions were collected directly into scintillation vials and counted after mixing with 9 ml of Aquasol (New England Nuclear Corporation, Boston, Mass.). The eluted compounds were detected at 254 nm by the Model 440 detector and quantitated using a Data Module. Nucleotides were identified by comparison with the retention times of known standards.

RESULTS

Tiazofurin is more inhibitory to CHO cells than ribavirin, giving comparable inhibition at one-tenth of the dose of the latter (Table 1). Phosphorylation of ribavirin by adenosine kinase has been shown to be necessary for its inhibitory activity (11). We have demonstrated the formation of tiazofurin-5′-monophosphate by CHO cell extracts, and it appears probable that this derivative is

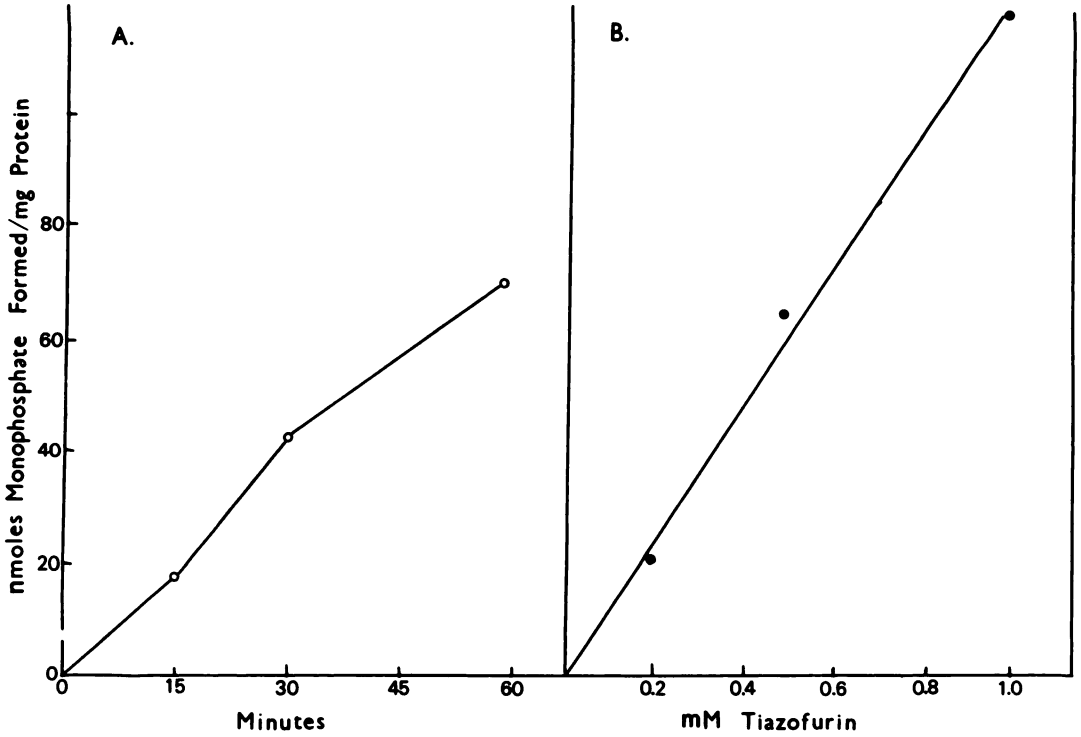

FIG. 2. *Phosphorylation of tiazofurin by a CHO cell extract as a function of time (A) and tiazofurin concentration (B)*
The procedure was as described for Table 2. For A, the tiazofurin concentration was 1 mM. For B, the incubation time was 60 min.

TABLE 3

Effect of tiazofurin on plating efficiency of CHO cells

The procedure is described in the text. The number of cells dispensed into each plate was taken as 100% plating efficiency. The results of duplicate determinations are shown.

Hours of incubation	Plating efficiency	
	No drug	Tiazofurin (100 μM)
0	0.76, 0.75	0.59, 0.74
1	0.77, 0.71	0.82, 0.78
4	0.64, 0.60	0.52, 0.59
24	0.52, 0.46	0.02, 0.02

the immediate precursor of the inhibitory NAD analogue (5, 6). From the structural similarities of tiazofurin, ribavirin, and pyrazofurin (Fig. 1) one might expect that they would be phosphorylated by the same enzyme; however, experiments with the adenosine kinase-deficient cell line, Rb^R-1, suggest that this is not the case (Tables 1 and 2). Line Rb^R-1 is highly resistant to ribavirin and pyrazofurin but demonstrates no resistance to inhibition by tiazofurin. Similarly, aC^R-7 cells, which are deficient in deoxycytidine kinase, show dramatic resistance to arabinosylcytosine but no resistance to RTC. The tiazofurin-resistant lines, RO^R-1 and RO^R-2, demonstrate no cross-resistance to either ribavirin or pyrazofurin. The data of Table 2 indicate that line Rb^R-1, as well as the parent CHO line, has the ability to phosphorylate tiazofurin. Formation of tiazofurin-5′-monophosphate by a crude extract is linear with time (Fig. 2A) and is a function of the concentration of tiazofurin (Fig. 2B).

The availability of the 5′-deoxy form of tiazofurin provides a convenient tool for the study of the importance of phosphorylation in the activity of tiazofurin. If phosphorylation at position 5′ is necessary for this compound to be toxic, as it is with ribavirin and pyrazofurin, the 5′-deoxy derivative should be inactive since phosphorylation cannot take place. This is, in fact, the case (Table 1). No toxicity to CHO cells was found with this compound at concentrations up to 500 μM. This provides further evidence that the 5′-hydroxyl group of tiazofurin is essential for inhibitory activity in these cells. Another derivative of tiazofurin, 2-α-D-ribofuranosylthiazole-4-carboxamide, was also tested (data not shown) and found to be completely inactive in this system.

Inhibition of CHO cell growth remains reversible for at least 4 hr (Table 3). After 24 hr in the presence of 100 μM tiazofurin, however, the plating efficiency dropped considerably, indicating a time requirement for cell death to occur. The incorporation of [^{3}H]uridine and [^{3}H]thymidine into perchloric acid-insoluble material of growing CHO cells was dramatically affected by the addition of tiazofurin to the cultures (Fig. 3). The incorporation of [^{3}H]leucine was inhibited only slightly, if at all, suggesting that the drug ultimately inhibits DNA and RNA synthesis but does not interfere with protein formation. These data are compatible with the observations of Table 4, which describe the inhibition, by tiazofurin and ribavirin, of the metabolism of [^{14}C]formate into total cellular purines. The selective inhibition of guanylate synthesis by tiazofurin is considerably stronger than that produced by ribavirin. This relationship is consistent with that of the growth-inhibitory activities of the two agents shown in Table 1, which could be taken to suggest that inhibition of guanylate synthesis is related to the mechanism of action of these agents. While the effect of guanylate synthesis was most dramatic, there also appeared to be a partial inhibition of adenylate synthesis, 50% by ribavirin and 68% by tiazofurin, suggesting that there could be slight inhibition at some point in *de novo* purine synthesis, or that the decreased guanine nucleotide levels have reduced the efficiency of adenylate synthesis from IMP since GTP is a required cofactor in this transformation.

The observation that tiazofurin selectively inhibits guanylate synthesis was verified by HPLC analysis of the nucleoside 5′-triphosphate pools of drug treated CHO

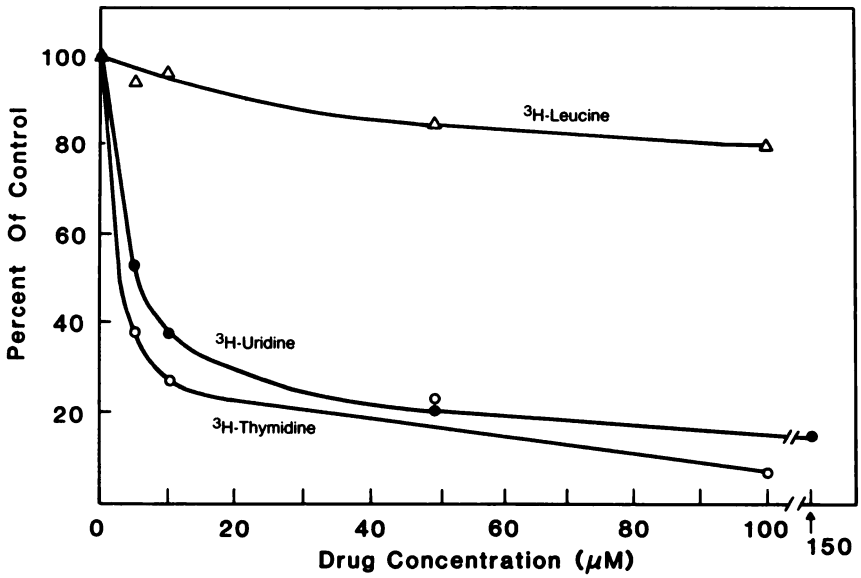

FIG. 3. *Effects of tiazofurin on the incorporation of radioactive precursors into perchloric acid-insoluble material*
The procedure is described under Materials and Methods.

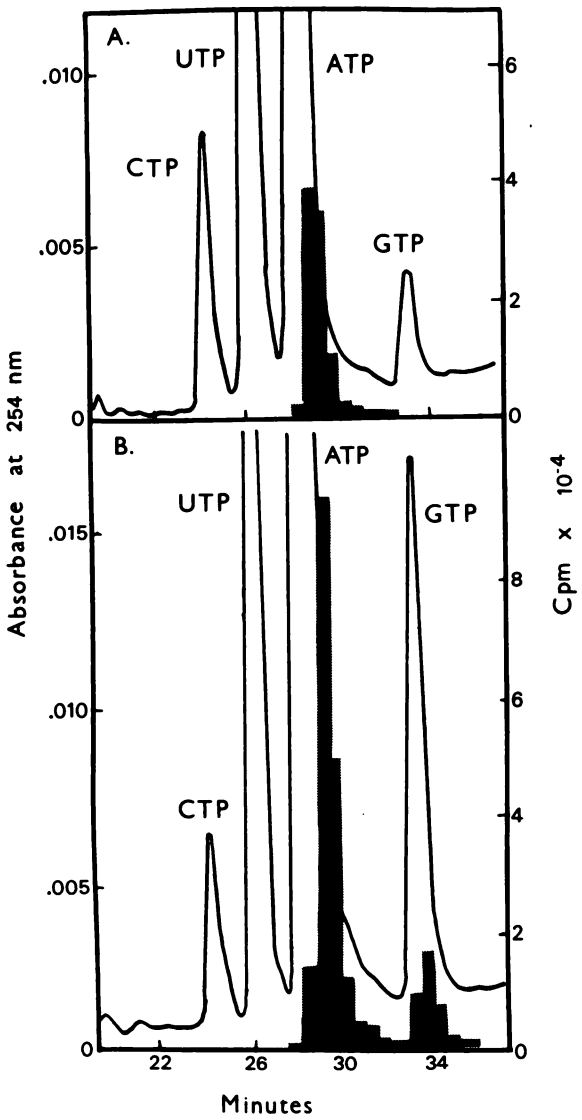

FIG. 4. *Effect of tiazofurin on the incorporation of [^{14}C]formate into cellular nucleotides*

The procedure is described under Materials and Methods. A, Nucleotide profile from 2.5 × 10^6 cells incubated for 5 hr in the presence of 100 μM tiazofurin. B, nucleotide profile from 2.5 × 10^6 cells incubated without drug. Each flask contained [^{14}C]formate (5 μCi/ml), which was added 1 hr after addition of drug.

cells (Table 5). In the presence of tiazofurin the GTP level dropped to 18% of the control while the ATP level decreased somewhat to 59%. Pyrimidine nucleotide levels, on the other hand, were elevated, CTP to 137% and UTP to 156% of the control. Figure 4B shows an HPLC analysis of ^{14}C formate incorporation into nucleotides of CHO cells in the absence of drug. Figure 4A is an analysis of an identical cell population incubated for 5 hr with tiazofurin. In the absence of tiazofurin, label from [^{14}C]formate appeared in both ATP and GTP (Fig. 4B). In the presence of drug, however, ATP was labeled but there was no detectable incorporation of isotope into GTP, indicating a complete selective block of GTP synthesis (Fig. 4A).

TABLE 5

Effect of tiazofurin on ribonucleoside 5′-triphosphate levels in CHO cells

Procedures were as described in the text. The tiazofurin concentration was 100 μM and incubation was for 5 hr.

Ribonucleoside 5′-triphosphate	Concentration in tiazofurin-treated cells
	% control
CTP	137
UTP	156
ATP	59
GTP	18

If the toxicity of tiazofurin does indeed reflect inhibition of IMP dehydrogenase, it should be possible to reverse or prevent the inhibition by providing the cells with an exogenous supply of guanine. Table 6 shows that guanine can reverse or prevent the action of tiazofurin, but relatively high concentrations are required. The HGPRT-deficient cell line, TG^R-3, was included in this experiment to determine whether or not phosphoribosylation of guanine is required for reversal. No reversal was observed with this cell line, indicating that guanine must be metabolized in order to relieve the inhibition of growth by tiazofurin.

DISCUSSION

The first step in the metabolism of tiazofurin to an active inhibitor appears to be phosphorylation at position 5′ by an enzyme other than adenosine kinase or deoxycytidine kinase. Both ribavirin and pyrazofurin require phosphorylation by adenosine kinase to be active. Although we have not yet obtained a tiazofurin-resistant cell line that is deficient in tiazofurin phosphorylation, this may reflect the possibility that the drug is phosphorylated by a kinase that is essential to cell growth (unlike the salvage kinases). From preliminary experiments it appears unlikely that pyrazofurin is phosphorylated by a phosphotransferase. Another possibility would be a nicotinamide nucleoside kinase; however, our attempts

TABLE 4

Drug effects of [^{14}C]formate incorporation into purines

CHO cells (2 × 10^6) were preincubated for 3 hr with 0.1 mM drug followed by 3 hr with [^{14}C]formate. Total cell nucleotides were extracted, hydrolyzed, and analyzed according to Materials and Methods.

Treatment (100 μM)	Radioactivity				
	Guanine	(%)	Hypoxanthine	Adenine	(%)
	cpm		*cpm*	*cpm*	
None	65,237	(100)	954	80,381	(100)
Ribavirin	5,198	(8)	970	39,779	(50)
Tiazofurin	290	(0.5)	794	25,302	(32)

TABLE 6

Reversal by guanine of tiazofurin inhibition of CHO cell clone formation

The procedures are described under Materials and Methods. Guanine was added directly to the medium prior to addition of cells. Growth was allowed for 8 days.

Guanine	Minimal inhibitory concentration of tiazofurin to cell lines	
	CHO	TG^R-3
μM	μM	
0	5.0	5.0
10	5.0	5.0
50	50.0	5.0
100	500.0	5.0

to detect such an enzyme in CHO extracts have been hampered by technical difficulties.

There are several lines of evidence suggesting that interference with guanylate synthesis may be the primary mechanism of action of tiazofurin. Among these are inhibition by tiazofurin of [^{14}C]formate incorporation into total cellular guanine nucleotides, decrease in cellular GTP pool levels after incubation with drug, and the ability of guanine to reverse the growth inhibitory effects of tiazofurin. The ability of tiazofurin to replace nicotinamide mononucleotide in the formation of NAD and the inhibitory activity of the resulting analogue to inosinate dehydrogenase have been documented (5, 6). Although IMP dehydrogenase has been implicated as a site of action of a number of purine analogues, in most cases the evidence is not strong indicating that perturbations of nucleotide levels within the cell are actually detrimental. Among the most positive correlations in this regard are the careful descriptions of mycophenolic acid action by Lowe *et al.* (12) and Cass *et al.* (13). More recently, Cohen *et al.* (14), working with mycophenolic acid in S-49 cells, reported results suggesting that GTP is required for DNA synthesis. Our observations with tiazofurin that the depletion of GTP rather than that of dGTP produces toxic effects are consistent with this suggestion.

It is interesting to note that, although the three compounds discussed in this report—tiazofurin, ribavirin, and pyrazofurin—share some similarities in structure, there are remarkable differences in their mechanisms of action. As already discussed, ribavirin and pyrazofurin are phosphorylated by adenosine kinase while tiazofurin is activated by another, yet unknown, kinase. The primary mechanism for action of ribavirin reportedly is the inhibition of IMP dehydrogenase by the monophosphate derivative (15). NAD analogue formation from ribavirin has not been reported. Pyrazofurin acts also at the monophosphate level; however, its target enzyme is orotidylate decarboxylase (EC 4.1.1.23) (16) and thus it inhibits pyrimidine synthesis. These results are in contrast with tiazofurin, which appears to act primarily through NAD analogue formation.

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