Biochemical Pharmacology, Vol. 31, No. 14, pp. 2371–2380, 1982.
Printed in Great Britain.

0006-2952/82/142371-10 $03.00/0
Pergamon Press Ltd.

INITIAL STUDIES ON THE MECHANISM OF ACTION OF A NEW ONCOLYTIC THIAZOLE NUCLEOSIDE, 2-β-D-RIBOFURANOSYLTHIAZOLE-4-CARBOXAMIDE (NSC 286193)

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(*Received* 3 *August* 1981; *accepted* 5 *January* 1982)

Abstract—Studies on the mechanism of action of a new oncolytic nucleoside, 2-β-D-ribofuranosylthiazole-4-carboxamide, have been undertaken using P388 murine leukemia cells growing in culture. The title compound was cytotoxic at micromolar levels, but a number of simple substitutions of both the ring and sugar moieties nullified cytotoxicity. Cytofluorimetric analysis revealed that the drug arrests cells in the "S phase" of the cell cycle. At antiproliferative concentrations, the agent inhibited the synthesis of both RNA and DNA. The macromolecular incorporation of preformed pyrimidines, including thymidine, was inhibited by the drug but, among the purines, this effect extended only to members of the adenine family and, in fact, the utilization of guanine and its congeners was reproducibly stimulated. When an examination was made of the ability of a comprehensive series of preformed purines and pyrimidines to overcome the inhibition of thymidine incorporation provoked by exposure to the thiazole nucleoside, the guanines were notably effective, but xanthosine also was shown to be an active antidote. Confirmation that the drug was producing a state of guanine deprivation was provided by high performance liquid chromatography (HPLC) analysis of acid-soluble extracts: a time-dependent fall in the concentrations of GMP and GTP ensued upon exposure to the drug; on the other hand, IMP concentrations increased by ~15-fold. Pursuant to these findings, an examination was made of the enzymologic steps unique to the biosynthesis of guanine nucleotides in cells exposed to cytotoxic concentrations of the drug. No prominent inhibition of GMP synthetase could be demonstrated *in vitro* or in culture, but the specific activity of IMP dehydrogenase underwent substantial reductions in both of these cases. HPLC analyses of extracts of cultures exposed to supralethal concentrations of the title compound provided evidence of modest anabolism to the 5′-monophosphate among other products; *in vitro* a chemically synthesized sample of 2-β-D-ribofuranosylthiazole-4-carboxamide-5′-monophosphate was twenty times more potent than the parent nucleoside in inhibiting IMP dehydrogenase. On kinetic analysis, this inhibition was non-competitive with IMP as the variable substrate.

The synthesis of a series of thiazole-C-nucleosides structurally related to the antiviral agent ribavirin was described by Fuertes *et al.* in 1976 [1] and Srivastava *et al.* in 1977 [2]. One of these, 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC 286193, hereinafter referred to as "thiazole nucleoside"), possessed significant antiviral activity against type 1 herpes virus, type 3 parainfluenza virus, and type 13 rhinovirus [2]. Subsequent to these findings, a systematic examination of the oncolytic properties of this novel thiazole nucleoside was undertaken at the National Cancer Institute [3]. It was found that the title compound exhibited notable antitumor activity against several murine leukemias including L1210 and P388. More importantly, mice bearing the Lewis lung carcinoma, a neoplasm refractory to many chemotherapeutic agents, were cured by treatment with the thiazole nucleoside over an unusually broad range of doses (25–800 mg/kg). The present studies were undertaken, then, to determine the mechanism of action of this new oncolytic agent.

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MATERIALS AND METHODS

Materials

RPMI 1640 medium and calf serum were obtained from HEM Research Inc., Rockville, MD. Radiochemicals, L-[U-^{14}C]valine (285 mCi/mmole) and [2-^{14}C]thymidine (51.6 mCi/mmole), were purchased from the New England Nuclear Corp., Boston, MA. [8-^{14}C]Adenosine (57 mCi/mmole), [8-^{3}H]deoxyadenosine (6.8 Ci/mmole), [8-^{14}C]deoxyguanosine (54.6 mCi/mmole), [U-^{14}C]guanosine (525 mCi/mmole), [8-^{14}C]hypoxanthine (52.8 mCi/mmole), [2-^{14}C]deoxycytidine (29.9 mCi/mmole), [2-^{14}C]deoxyuridine (39.4 mCi/mmole), [^{14}C]formic acid (58 mCi/mmole), [8-^{14}C]inosine (50 mCi/mmole) and L-[U-^{14}C]glutamine (50 mCi/mmole) were products of the Amersham Corp., Arlington Heights, IL. All other chemicals were of the highest purity available. The thiazole nucleoside and its con-

geners, except for compounds 3, 4, 10 and 11 (Fig. 2), were synthesized according to the method of Srivastava *et al.* [2]. Methods for synthesis of compounds 3, 10 and 11 (Fig. 2) are to be published elsewhere (P. C. Srivastava and R. K. Robins). Compound 4 (Fig. 2) was synthesized and supplied by Prof. Louis Malspeis, Ohio State University, Columbus, OH.

Preparation of [2,8-^{3}H]*IMP.*

Twenty mCi of [2,8-^{3}H]hypoxanthine was lyophilized and then reconstituted in 1 ml of water; 5-phosphoribosyl-1-pyrophosphate (PRPP)-$MgCl_2$ was added to a final concentration of 10 mM, and the pH was adjusted to 8.0. An equal volume of partially purified hypoxanthine guanine phosphoribosyl transferase from mouse brain [4] (0.18 units) was added, and the mixture was incubated at 37° for 60 min. Protein was precipitated by the addition of 1/10th volume of 1 N HCl. Following centrifugation at 12,000 *g* for 6 min, the clear supernatant fraction was spotted as a strip 10 cm from the bottom of a 45 × 57 cm sheet of Whatman 3MM paper and subjected to ascending chromatography in a solvent composed of ethanol–1 M ammonium acetate (70:30, v/v); the spot of IMP was visualized with ultraviolet light, excised, and eluted with water. After two lyophilizations, the product was dissolved in water, and sufficient nonradioactive IMP was added to bring the final specific activity to 2195.5 mCi/mmole.

Cytotoxicity studies

P388 cells in log phase growth (1–5 × 10^5 cells/ml) in 5 ml of culture medium (RPMI 1640 containing 10% calf serum, 2 mM L-glutamine and 5 μM 2-mercaptoethanol) were incubated for 24 or 48 hr at 37° in an atmosphere of 95% air and 5% CO_2, with saline, thiazole nucleoside or its congeners at concentrations ranging from 0.1 to 1000 μM, or as indicated in the appropriate legends. Cells were counted in a Coulter counter; under the conditions used, there was a 2 to 4-fold increase in cell number in control flasks in 24–48 hr.

To study the influence of various nucleosides and bases on the reversal and/or prevention of cytotoxicity exerted by the thiazole nucleoside, P388 cells (1–5 × 10^5 cells/ml, 5 ml/flask) were incubated with nucleoside or base (10–100 μM) or saline and thiazole nucleoside (0.75–2.0 μM) or saline, at 37° for 24 or 48 hr in an atmosphere of 95% air and 5% CO_2. The cells were counted at both 24- and 48-hr time points.

Effect of the thiazole nucleoside on the cell cycle

P388 cells in exponential growth were continuously exposed to 100 μM thiazole nucleoside for 24 hr. Aliquots were removed at various time intervals and processed for flow cytofluorometry using the hypotonic citrate/propidium iodide method described by Krishan [5]. DNA distribution curves were generated using a model 4801 Cytofluorograf (Ortho Instruments) coupled with a Northern Scientific (model NS-633) pulse height analyzer. The DNA histograms were normalized to unit area and plotted by means of a microcomputer. For recovery studies, cells were centrifuged at 24 hr after treatment with drug or saline and then washed once with Hanks' balanced salt solution. Cells were next suspended in fresh medium and incubated for 51 hr. Aliquots for cell counting and cytofluorography were sampled at various time intervals and processed as described earlier.

Effect of thiazole nucleoside on macromolecular synthesis

P388 cells (2 × 10^7 cells/ml, 5 ml/flask) in culture medium were incubated with 0.1 mM thiazole nucleoside, or with 50 μl saline at 37°. After 2 hr, 10 μCi/flask of L-[U-^{14}C]valine, [2-^{14}C]uridine or [2-^{14}C]thymidine was added, and incubations were continued for 30 min. The cells were then quickly centrifuged and precipitated with 1 ml of 5% perchloric acid (PCA). The precipitates were washed four times with 1.5 ml of cold 5% PCA, digested in 50 μl of 40% KOH at 95°, neutralized with glacial acetic acid, and counted by liquid scintillation spectrometry.

Effect of nucleosides and bases on the inhibition of DNA synthesis produced by the thiazole nucleoside

P388 cells (1 × 10^7 cells/ml, 4 ml/flask) in culture medium were incubated with various precursors at a final concentration of 50 μM or with saline and the thiazole nucleoside (5 μM), for 2 hr at 37°; [2-^{14}C]thymidine (2.5 μCi/flask) was then added, and incubations were continued for 30 min. Cells were quickly centrifuged, precipitated, and processed as detailed above.

Influence of the thiazole nucleoside on the incorporation of radioactive nucleosides into macromolecules

To 4 ml of P388 cells (1 × 10^7 cells/ml) in culture, drug was added to make a 5 μM final concentration; the mixture was incubated for 2 hr at 37°. One of the radiolabeled nucleosides (5 μCi) listed in Table 2 was then added to each flask, and the incubations were continued for a further 30 min. The cells were then processed as detailed earlier.

Effect of thiazole nucleoside treatment on the concentrations of nucleoside phosphates

P388 cells (1 × 10^7 cells/ml) in culture medium were incubated with saline or thiazole nucleoside (final concentration 3.5 μM) for 2, 4 or 24 hr at 37°. Cells were separated by centrifugation and precipitated with 1 ml of 5% PCA. PCA supernatant fractions were quickly neutralized to pH 6.0, and an aliquot was loaded on a radial compression column of Partisil-10 SAX resin (Waters Co., Milford, MA) pre-equilibrated with 0.05 M ammonium phosphate buffer, pH 2.88. After loading the sample, the column was eluted isocratically with the starting buffer for 10 min and then developed with a linear gradient of 0.05 M ammonium phosphate, pH 2.88, and 0.25 M ammonium phosphate, pH 4.50, for 30 min to elute nucleoside monophosphates. Nucleoside triphosphates were determined by anion exchange high performance liquid chromatography (HPLC) as described in the legend to Table 2.

Effect of the thiazole nucleoside on the incorporation of [8-^{14}C]*hypoxanthine into purine nucleoside monophosphates*

P388 cells in culture were treated with 10 μM thia-

zole nucleoside or saline for 90 min; 2.5 μCi of [8-^{14}C]hypoxanthine was then added, and incubations were continued for 30 min. The cells were quickly centrifuged and precipitated with 1 ml of 5% PCA. The PCA supernatant fraction was immediately neutralized to pH 6.0 with 40% KOH, centrifuged at 12,000 *g* for 1 min to remove potassium perchlorate, and the supernatant fraction analyzed by HPLC as detailed above.

Effect of the thiazole nucleoside on IMP dehydrogenase and GMP synthetase activities in culture and in vitro

In culture. P388 cells (1×10^7 cells/ml) were treated with saline or thiazole nucleoside (final concentration 5 μM) for 2 hr at 37°. After centrifugation, cells were washed twice with Hanks' balanced salt solution (HBSS). Cells were then suspended in 200 μl of 0.1 M Tris buffer (pH 7.5), containing 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 30% glycerol and 2 mM dithiothreitol, and freeze-thawed three times. The resulting suspension was then centrifuged for 3 min at 12,000 *g* and the supernatant fraction was passed through a column of Sephadex G-25 equilibrated with 0.05 M Tris buffer (pH 7.6), containing 2 mM dithiothreitol. The protein fraction was collected and used for the determination of enzyme activities.

In vitro. P388 cells in exponential growth were harvested by centrifugation and washed thrice with HBSS. The cell pellet (1×10^8 cells/ml) was homogenized as described above. Reaction mixtures for measuring IMP dehydrogenase contained: 5 μl of [2,8-^{3}H]IMP (365 μCi/ml, 2.2 Ci/mmole) containing 1 mM NAD; 5 μl of 0.5 M KCl containing 8 mM uridine and 2.6 μM allopurinol at pH 8.0; 5 μl of either 0.01 M Tris–HCl, pH 8.0, or thiazole nucleoside in buffer; and 5 μl of cell extract. Control reaction vessels (blanks) received 5 μl of 100 μM mycophenolic acid. The reaction was started by a brief centrifugation to admix the components; after 15 min at 37°, the reaction was terminated by heating at 95° for 1 min; 5 μl of the centrifuged reaction mix was spotted on Whatman 3MM paper, overspotted with 5 μl of a mixture of IMP and XMP (10 mM each), and subjected to paper electrophoresis at 3000 V for 60 min in 0.05 M sodium phosphate buffer, pH 7.2. Spots corresponding to XMP were excised and eluted with water, and their radioactivity was measured by liquid scintillation spectrometry. Under the conditions used, mycophenolic acid reduced the radioactivity found in the XMP spot from ~50,000 cpm to ~1000 cpm.

GMP synthetase activity was measured by incubating 5 μl of [8-^{14}C]XMP (25 μCi/ml, 50 mCi/mmole), 5 μl of 0.05 M L-glutamine or water, 5 μl of 0.02 M ATP-$MgCl_2$ in 0.05 M Tris–HCl (pH 7.8), or buffer, 5 μl of water or thiazole nucleoside and 5 μl of cell extract. The reaction was started by accelerating the components to 12,000 *g* for 10 sec; after 30 min at 37°, the reaction was terminated by heating at 95° for 2 min; 5 μl aliquots of the centrifuged reaction mix were spotted on Whatman 3MM paper, overspotted with 5 μl of a mixture of XMP and GMP (10 mM each) and subjected to paper electrophoresis for 60 min, in 0.05 M sodium phosphate buffer, pH 7.2. Spots corresponding to GMP were excised and eluted with water, and their radioactivities were determined by liquid scintillation spectrometry [6].

Enzyme activities are expressed as nmoles of product formed per mg protein per hr.

RESULTS

Relationship of structure to cytotoxicity of thiazole nucleoside and its congeners

Figure 1 illustrates the cytotoxic potency of the title compound (Fig. 2, No. 1). The drug exhibited an IC_{50} of ~1.6 μM while the free base, thiazole carboxamide (Fig. 2, No. 2), was devoid of cytotoxicity. The 5'-monophosphate of the thiazole nucleoside (Fig. 1 and Fig. 2, No. 3) was less potent than the title nucleoside (IC_{50} ~3.75 μM). A carboxamide moiety in the 4-position appears to have been essential for cytotoxicity since substitution by carboxylic acid (Fig. 2, No. 4), thiazole-4-carboxamide (Fig. 2, No. 5), or thiocarboxamide (Fig. 2, No. 6) or a change of the carboxamide to the 5-position (Fig. 2, No. 7) resulted in a loss of activity. Substitution of the 5'-hydroxyl moiety either with hydrogen (Fig. 2, No. 8) or with iodine (Fig. 2, No. 9) rendered the molecule inert. Replacement of oxygen for carbon in the 5-position, and nitrogen for sulfur in the heterocyclic ring (Fig. 2, No. 10), also resulted in the loss of cytotoxicity. Similarly, substitution of a deoxyribosyl moiety for the ribosyl moiety (Fig. 2, No. 11) rendered the molecule non-toxic to P388 cells in culture.

Effect of thiazole nucleoside on the cell cycle

The upper panels of Fig. 3 show the progressive changes in the cell cycle distribution of P388 cells that were exposed continuously for 24 hr to a 100 μM concentration of the thiazole nucleoside. There was a marked increase in the early "S" phase with a concomitant decrease in the late "S" and "G_2 + M"

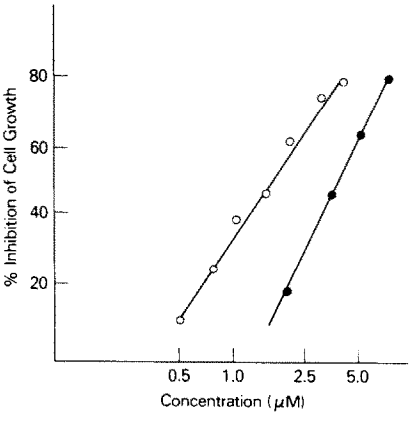

Fig. 1. Cytotoxicity of thiazole nucleoside and a congener to P388 cells in culture. P388 cells in culture were incubated with the drug, the analog, or saline. Control values and experimental conditions are described in Materials and Methods. Key: (○—○) thiazole nucleoside (Fig. 2, No. 1), and (●—●) thiazole nucleoside 5'-monophosphate (Fig. 2, No. 3).

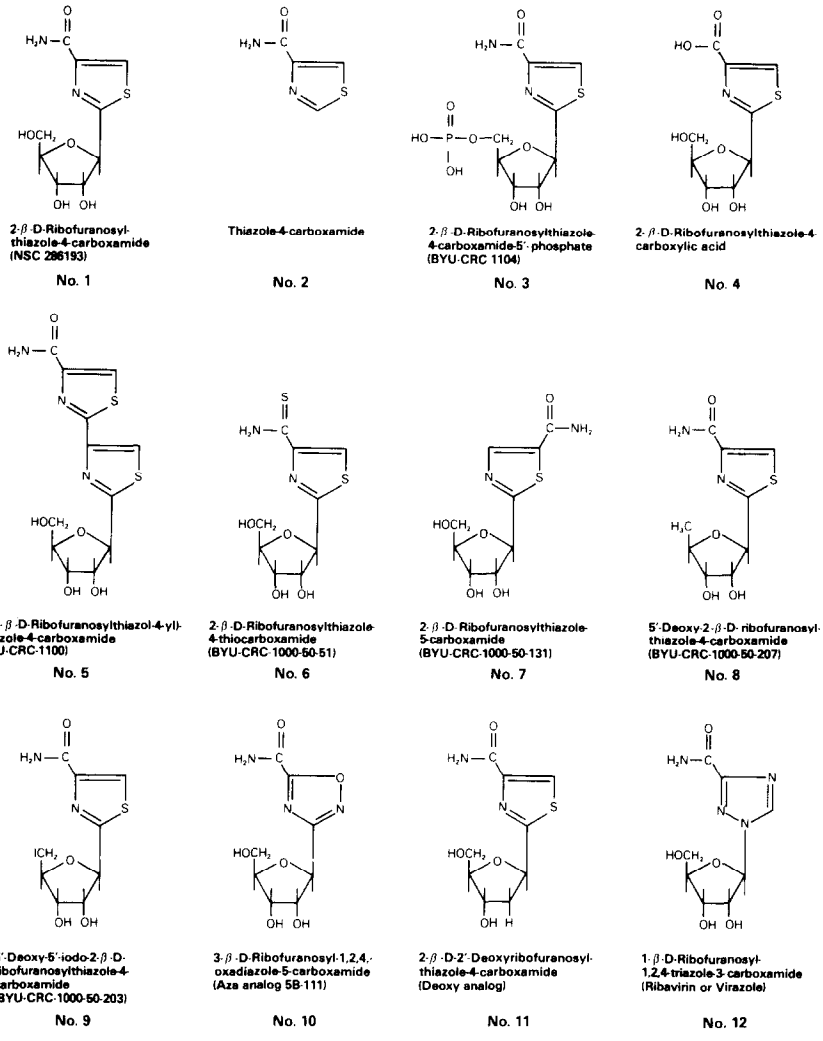

Fig. 2. Structures of the thiazole nucleoside and its analogs.

portion of the curve at 6 hr. By 12 hr the population was predominantly in mid to late "S" phase. At 15 hr, a portion of the cells had re-entered the "G_1" compartment (data not shown in Fig. 3). By 24 hr, additional cells had entered the "G_1" and early "S" phase compartments and the resultant curve was similar to the 6-hr time point. These studies indicate that the thiazole nucleoside interrupted the cell cycle at the "S" phase.

Characterization of cytotoxicity of the thiazole nucleoside

To determine whether the title compound was cytostatic or cytocidal, cultures of P388 cells were exposed to an IC_{90} concentration of the drug (100 μM) for 24 hr, at which point the cells were washed, the medium was changed, and the rate of replication was monitored by Coulter counting and cell-cycle progression analysis (Figs. 3 and 4). Under these conditions, removal of the drug permitted the resumption of DNA synthesis within 6 hr as evidenced by the increase of cells in the late "S" and "G_2 + M" phases and the concomitant decrease of cells arrested in the "G_1" phase at this time (Fig. 3, lower panels). However, as indicated in Fig. 4, cells plated in drug-free medium after 24 hr treatment proliferated at a lesser rate than their untreated counterparts. Thus, the doubling time of untreated cells was 14 hr and that of rescued cells was 45 hr, a rate similar to that of cells during drug exposure. This retardation of growth is possibly attributable to the continued intracellular presence of the drug or its metabolites. Despite the depression in doubling time, these studies demonstrate some resumption of growth upon drug removal and therefore suggest that the thiazole nucleoside was exerting a partially cytostatic effect. However, the curative therapeutic potency of the compound *in vivo* against, for example, the Lewis Lung carcinoma also implicates true cell-killing under other experimental conditions.

Studies on the mechanism of action of the thiazole nucleoside: macromolecular incorporation studies

As an initial step in the investigation of the mechanism of action of the thiazole nucleoside, its influ-

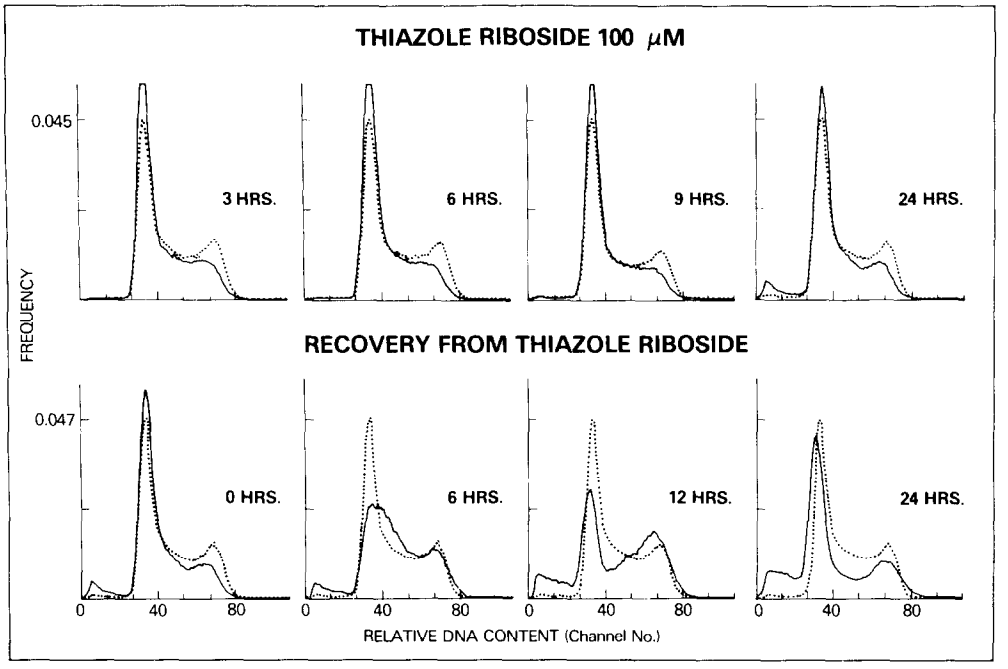

Fig. 3. DNA histograms of P388 cells treated continuously with the thiazole nucleoside (100 μM) for 24 hr and their recovery after drug removal. Details are given in Materials and Methods. Ordinate: frequency of a given DNA content expressed as a fraction of unit area. Abscissa: relative DNA content. Dotted lines refer to control and solid lines refer to thiazole nucleoside treatment. Upper panels refer to the cells incubated with thiazole nucleoside for 24 hr, and the lower panels refer to the recovery of cells following removal of the drug after 24 hr of treatment and incubation in fresh medium.

ence on the incorporation of metabolic precursors into RNA, DNA or protein was examined using P388 cells growing in culture. Figure 5 documents that the drug (100 μM) had only a minor effect on the incorporation of L-valine into protein but nearly equipotently arrested the incorporation of thymidine and uridine into nucleic acids.

In corollary studies, cells were incubated with the drug for 2 hr and sampled at various times to examine the onset of inhibition of DNA synthesis (Fig. 6), approximately 30 min elapsed before this process began to be curtailed.

The influence of the title compound on the incorporation of labeled precursors into nucleic acids was studied next (Table 1). The drug inhibited the incorporation of all bases and nucleosides examined,

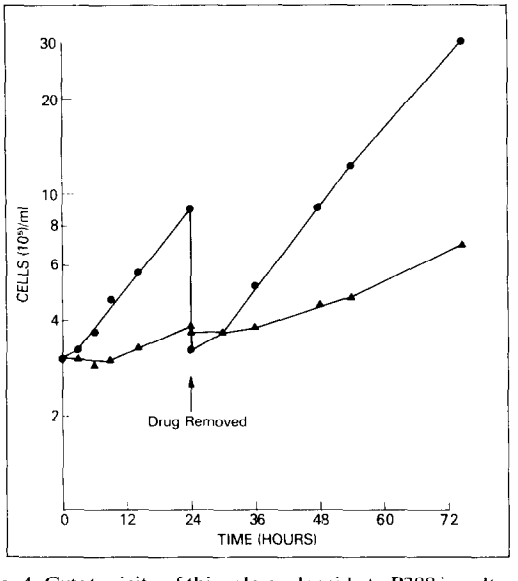

Fig. 4. Cytotoxicity of thiazole nucleoside to P388 in culture and the recovery of cells after 24 hr of drug treatment. P388 cells were incubated with the drug (100 μM final concentration) (▲) or with saline (●) for 24 hr. Cells were then centrifuged, washed once with Hanks' balanced salt solution, and cultured in fresh medium. The cell proliferation was monitored for 51 hr.

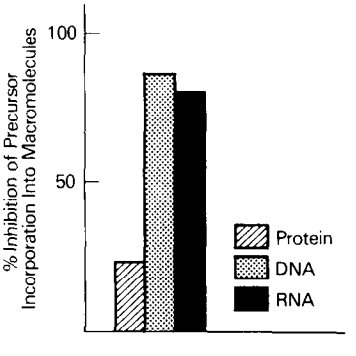

Fig. 5. Influence of thiazole nucleoside on macromolecular synthesis by P388 cells in culture. P388 cells were incubated with the drug (100 μM final concentration) or saline and processed as described in Materials and Methods. In control cells, 0.09, 40.0 and 12.4 pmoles of labeled valine, thymidine and uridine were incorporated per 10^6 cells respectively.

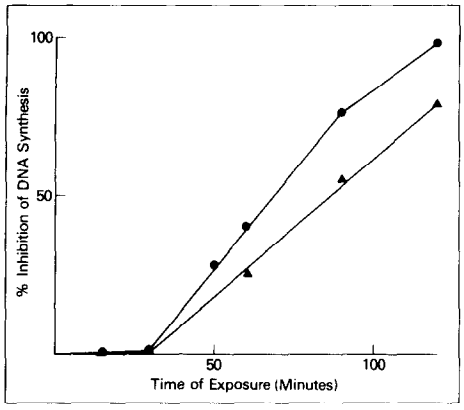

Fig. 6. Influence of thiazole nucleoside treatment on the synthesis of DNA by P388 cells. P388 cells (1×10^6 cells/ml, 5 ml) were incubated with 5 μM (▲—▲) or 10 μM (●—●) thiazole nucleoside and pulsed with 2.5 μCi of [^{14}C]thymidine at time zero. Aliquots of the cell suspension were taken at the times indicated for the measurement of DNA synthesis, as described in Materials and Methods. Inhibitions were calculated by comparison to the incorporation rates measured in control flasks receiving saline. In control cells, 40.0 pmoles of labeled thymidine was incorporated per 10^6 cells.

except for those belonging to the guanine family. Indeed, the incorporation of guanosine and deoxyguanosine was even stimulated in the presence of the thiazole nucleoside.

Perturbation of nucleotide concentrations

The foregoing results suggest that the thiazole nucleoside might produce a state of guanine deprivation. To examine this hypothesis, P388 cells in culture were exposed to the drug for 2, 4 or 24 hr and the profile of their acid-soluble nucleotides was measured by HPLC. GTP concentrations were selectively reduced after 2 and 4 hr of exposure to 3.5 μM thiazole nucleoside, but after 24 hr all the nucleoside triphosphate pools were drastically reduced (Table 2). The effect of the drug on nucleoside monophosphate pools was next examined (Fig. 7).

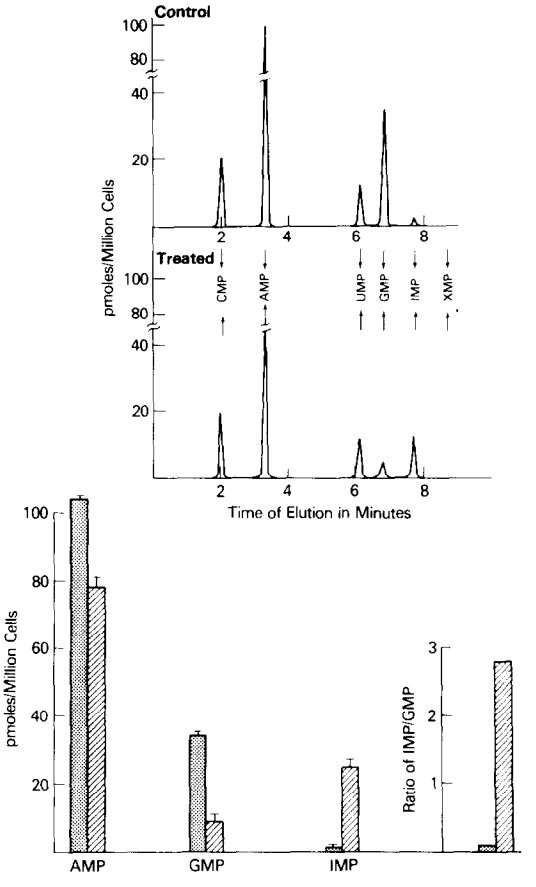

Fig. 7. Perturbation of nucleoside monophosphate concentrations by thiazole nucleoside. P388 cells in culture were incubated with 10 μM drug or saline for 2 hr and processed as detailed in Materials and Methods. Key: (▩) control and (▨) thiazole nucleoside treatment.

Table 1. Influence of the thiazole nucleoside (TR) on the incorporation of labeled precursors into the macromolecules of P388 cells in culture*

Precursor	Precursor incorporated (pmoles/10^6 cells) Control	+TR	Percent change of incorporation of precursors by drug treatment
[8-^{14}C]Adenosine	57.1	36.0	−37
[8-^{3}H]Deoxyadenosine	0.84	0.41	−49
[8-^{14}C]Inosine	64.0	22.9	−64
[8-^{14}C]Hypoxanthine	66.4	35.8	−54
[U-^{14}C]Guanosine	15.2	19.8	+130
[8-^{14}C]Deoxyguanosine	74.5	104.3	+140
[2-^{14}C]Uridine	125.1	61.3	−51
[2-^{14}C]Deoxyuridine	16.1	2.6	−84
[2-^{14}C]Deoxycytidine	35.1	14.3	−58
[2-^{14}C]Thymidine	51.9	10.4	−80

* P388 cells were incubated with 5 μM thiazole nucleoside or saline and processed as detailed in Materials and Methods. The values are averages of duplicate determinations from one experiment.

Table 2. Influence of thiazole nucleoside (TR) and/or nucleosides on the concentration of nucleoside triphosphates in P388 cells in culture*

Treatment	Time after treatment (hr)	Nucleoside triphosphate concentration (% of control)			
		CTP	UTP	ATP	GTP
TR, 3.5 μM	2	93	94	64	32
	4	77	117	77	33
	24	46	59	41	26
Guanosine, 50 μM	2	73	69	73	119
	24	76	71	80	74
Guanosine + TR	2	95	69	81	117
	24	41	49	30	24
Deoxyguanosine, 50 μM	2	86	75	82	122
	24	61	56	63	67
Deoxyguanosine + TR	2	82	82	79	175
	24	28	36	23	16
Xanthosine, 50 μM	2	59	66	79	58
	24	69	58	66	62
Xanthosine + TR	2	82	109	52	64
	24	22	24	20	19
Thymidine, 50 μM	2	73	79	82	64
	24	30	27	28	39
Thymidine + TR	2	82	91	60	47
	24	19	18	13	18

* P388 cells (4×10^7 cells/flask) were incubated with 3.5 μM thiazole nucleoside or saline with or without 50 μM nucleoside for 2, 4 or 24 hr, and PCA extracts were prepared as described under Materials and Methods. Nucleoside triphosphates were measured by HPLC using an Altex NH_2 column (0.46 × 25 cm) and isocratic elution with 0.3 M KH_2PO_4, pH 3.8–2% (v/v) acetonitrile at a flow rate of 4 ml/min. The retention times of CTP, UTP, ATP and GTP were 8.2, 9.2, 14.7 and 28.3 min respectively. In control cells, levels of CTP, UTP, ATP and GTP were 0.22, 0.53, 1.31 and 0.36 nmoles/10^6 cells, respectively (average of six determinations). The values are the averages of duplicate determinations from two experiments (N = 4).

While AMP concentrations were reduced by 18%*, there was a dramatic decrease in the concentration of GMP (74%). Furthermore, the concentration of IMP increased ~15-fold (XMP could not be detected in these extracts whether or not the cells had been treated with the thiazole nucleoside).

In further experiments, the incorporation of radiolabeled hypoxanthine into GMP was found to be almost totally inhibited (94%) by the drug, while its incorporation into AMP was depressed by 57%*. At the same time, incorporation of hypoxanthine into IMP was potentiated 10-fold (Table 3).

Reversal studies

The foregoing results prompted attempts to reverse the effects of the thiazole nucleoside with bases or nucleosides of the guanine family. As Table 4 documents, guanine, guanosine, deoxyguanosine and xanthosine were able to correct entirely the depression of thymidine incorporation provoked by a 2-hr exposure to the title compound. In addition, at 2 hr, deoxyguanosine and guanosine were able to rescue drug-mediated depression of GTP pools (Table 2). This biochemical finding suggested that these counteragents might prevent the cytotoxicity of the drug. In fact, however, none of the nucleic acid constituents listed in Table 5 proved capable of overcoming the antiproliferative effects of the thiazole nucleoside to cells in culture. This failure might have been a result of the longer exposure times (1–2 days) used in the cell culture studies or to some peculiarity of the P388 leukemia cell line†. Also explanatory of this failure was the finding that none of the bases or nucleotides, when incubated with P388 cells in the presence of the drug, was able to overcome the depression in GTP pools engendered by it over a 24-hr period (Table 2). Moreover, as

Table 3. Influence of thiazole nucleoside treatment on the incorporation of [8-^{14}C]hypoxanthine into purine nucleoside monophosphates*

Nucleotide	Hypoxanthine incorporation (pCi/10^6 cells)		% of Control
	Treatment		
	Saline	Drug	
AMP	3719 ± 26	1599 ± 220	43
GMP	948 ± 2	57 ± 16	6
IMP	128 ± 6	1313 ± 44	1030

* P388 cells in culture were treated with 10 μM thiazole nucleoside or saline for 90 min; 2.5 μCi of [8-^{14}C]hypoxanthine was added and, after an additional 30 min, nucleoside monophosphates were extracted and analyzed as described in Materials and Methods.

* The depression in the biosynthesis of AMP and ATP may have resulted from the observed depression of GTP, since GTP is an obligatory cosubstrate, with IMP, in the biosynthesis of adenylosuccinic acid and, so, of AMP.

† We have observed that 50 μM guanosine can partially overcome the cytotoxicity of thiazole nucleoside to Lewis lung carcinoma cells in culture.

exemplified by thymidine, many of these agents themselves depressed the nucleoside triphosphate pools to a marked degree.

Enzymologic locus of action of thiazole nucleoside

Because of the fact that the title compound produced such a distinct depression in the pool sizes of GMP and GTP, it seemed reasonable that it might have been acting at one or both of the enzymic steps unique to the biosynthesis of guanine nucleotides, i.e. by inhibiting IMP dehydrogenase and/or GMP synthetase.

In vitro the thiazole nucleoside, at a concentration of 12 mM, potently inhibited IMP dehydrogenase but exerted only marginal inhibition of GMP synthetase (Table 6). Similarly, when the drug was incubated with P388 cells in culture, only IMP dehydrogenase activity was strongly inhibited. Exhaustive dialysis fully reversed this inhibition (data not shown). It is worthwhile stressing that, in culture, 5 μM drug produced enzyme inhibition equivalent to that produced by 12 mM drug *in vitro*. This discrepancy suggests that the drug might have been metabolized to a more active enzyme-inhibitory species.

To examine the possible metabolism of the title compound further, P388 cells in culture were incubated with saline or the drug at a concentration of 10 mM for 2 hr, and their nucleotide pools were examined by HPLC (Fig. 8). Several definite peaks

Table 4. Influence of various precursors on the inhibition by the thiazole nucleoside of radioactive thymidine incorporation in cultured P388 cells*

Precursor	Percent inhibition of DNA synthesis in the presence of drug and precursor compared to that seen in the presence of drug alone
Adenine	83
Adenosine	76
Xanthine	81
Inosine	53
Deoxyinosine	83
Hypoxanthine	79
Cytosine	62
Cytidine	61
Uracil	59
Uridine	59
Guanine	8
Guanosine	0
Deoxyguanosine	0
Xanthosine	0

* To the P388 cells in culture, 50 μM concentrations of bases or nucleosides were added together with 5 μM thiazole nucleoside or saline for 2 hr and processed as detailed in Materials and Methods. Under these conditions 10^6 cells incorporated 40.0 and 7.0 pmoles of [2-^{14}C]thymidine in the absence and presence of 5 μM thiazole nucleoside respectively. The values are averages of duplicate determinations from one experiment.

Table 5. Influence of various precursors in nullifying the cytotoxicity of the thiazole nucleoside to P388 cells in culture*

Precursor	% Reversal/prevention of cytotoxicity of the thiazole nucleoside	Toxicity of precursor alone at 100 μM (% depression of cell count)
Purine		
Adenine	<10	<10
Adenosine	<10	<10
Guanine	<10	<10
Guanosine	<10	12
Hypoxanthine	<10	<10
Aminoimidazolecarboxamide-ribonucleotide (AICAR)	<10	<10
Deoxyadenosine	<10	<10
Deoxyinosine	<10	<10
Deoxyguanosine	<10	71
Pyrimidine		
Thymine	<10†	<10
Thymidine	<10†	69
Uracil	<10	<10
Uridine	<10	<10
Cytosine	<10	<10
Cytidine	<10	<10
Deoxyuridine	<10	<10
Deoxycytidine	<10	<10
L-Glutamine‡	<10	<10
Nicotinamide	<10	30

* To 5 ml of cell suspension (0.1–0.5 × 10^6/ml) were added: 50 μl of nucleoside, base (final concentration of 10–100 μM) or saline and 50 μl of thiazole nucleoside (final concentration of 1 μM) or saline; the cells were incubated at 37° for 24 or 48 hr in an atmosphere of 95% air and 5% CO_2. After the incubation, 0.5 ml aliquots were taken for cell counts. Control values and experimental conditions are described more fully in Materials and Methods.

† Thymine and thymidine at a concentration of 50 μM produced 50 and 100% reversal/prevention, respectively, of the cytotoxicity of the thiazole nucleoside in two experiments out of five (not shown) but no reversal in three other experiments (shown).

‡ Tested in medium containing 0.01 mM L-glutamine instead of 2 mM L-glutamine. All other conditions were the same as detailed in Materials and Methods.

Table 6. Influence of thiazole nucleoside treatment on the activities of enzymes synthesizing GMP*

Enzyme	Enzyme activity (% inhibition)	
	Treatment in culture (5 μM)	Cell-free system (12,000 μM)
IMP dehydrogenase	80†	78†
GMP synthetase	17	11

* Assays were performed as detailed in Materials and Methods. In control cells, the level of IMP dehydrogenase activity was 257.0 ± 13.7 pmoles per mg protein per hr. The values are duplicate determinations from two experiments (N = 4).
† $P < 0.01$.

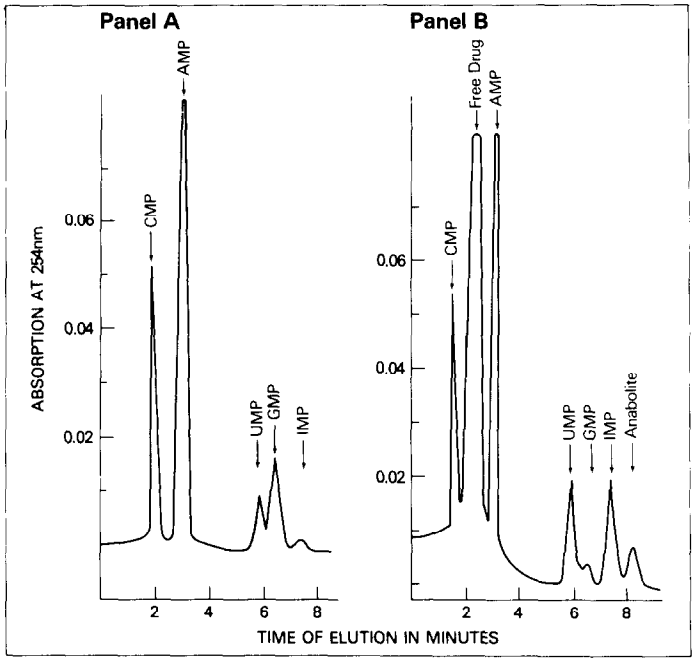

Fig. 8. Metabolism of thiazole nucleoside by P388 cells in culture. P388 cells were incubated with 10 mM drug or saline for 2 hr and analyzed as detailed in Materials and Methods. The 5'-monophosphate of the title compound eluted at 8.7 min and exhibited an absorption maximum at 236 nm.

unique to thiazole-treated cells were observed. The principal peak eluted at 8.7 min and corresponded to that of a sample of chemically synthesized thiazole nucleoside-5'-monophosphate (Fig. 2, No. 3). The compound exhibited a λ_{max} at 236 nm, identical to that of the authentic thiazole nucleoside-5'-monophosphate.* Finally, while the thiazole nucleoside was not a potent inhibitor of IMP dehydrogenase *in vitro* ($K_i = 8.2$ mM), its monophosphate derivative was considerably more effective ($K_i = 0.46$ mM). With IMP as the variable substrate, this inhibition was of the non-competitive type (not shown).

DISCUSSION

The structural relationship of the title compound to ribavirin (Fig. 2, No. 12) was noted by Srivastava *et al.*, who observed that all active antiviral nucleosides of this family of agents also inhibit guanine nucleotide biosynthesis [2, 7]; this relationship has also been documented by other workers [8]. Like ribavirin, the thiazole nucleoside also interrupts the incorporation of radiolabeled hypoxanthine into the guanine, but not adenine nucleotides of Ehrlich ascites tumor cells [2].

Several lines of evidence suggest that the thiazole nucleoside also produces its cytotoxic effects on P388 cells in culture by interrupting the biosynthesis of guanine nucleotides. The most compelling such evidence is the marked depression of the concentration of GMP and GTP seen in the treated cells shortly after their exposure to the drug. This evidence is strengthened by studies with radiolabeled precursors of nucleic acids. Of the comprehensive group of bases and nucleosides examined, only the members of the guanine family were incorporated at or above

* At least three other novel peaks were detected in the region of the chromatogram that contained di- and triphosphates. The identity of these peaks is under study.

control rates in the presence of the thiazole nucleoside. This phenomenon is most readily explained by a drug-induced state of guanine deprivation. Additionally, guanine and xanthine nucleosides were uniquely capable of counteracting the inhibition of the incorporation of labeled thymidine seen in the cells treated with the title compound *in vitro*. Still unexplained is the failure of guanine or guanosine to prevent and/or reverse the cytotoxicity to the P388 cells of the thiazole nucleoside. By contrast, the cytotoxicity of ribavirin was reported to be reversed by both guanosine and xanthosine [9]. However, because of the intrinsic cytotoxicity of these purines towards P388 cells, this failure might have had a technical basis related to the narrow therapeutic (or prophylactic) index of guanosine and xanthosine.

Although there are at least two enzymologic steps unique to the biosynthesis of guanine nucleotides, it seems clear that the title compound was principally acting at the level of IMP dehydrogenase. Strong support for this conclusion came from the finding that treatment with the drug engendered inhibition of this enzyme but not of GMP synthetase. Additionally, the concentration of IMP was elevated up to 15-fold in treated cells, with no apparent expansion of the XMP pool, as would be expected from blockade of GMP synthetase. Lastly, the incorporation of [^{14}C]hypoxanthine into guanine nucleotides was strongly inhibited by the thiazole nucleoside, and this was accompanied by an expansion of the [^{14}C]IMP pool.

Although the exact molecular species responsible for the inhibition of IMP dehydrogenase has not been identified in the present study, the parent drug is not a prominent candidate for this role. *In vitro* at a concentration of 8.2 mM, the thiazole nucleoside inhibited a crude preparation of the enzyme from cultured P388 cells only by 50%. Since the IC_{50} of the thiazole nucleoside was ~1 μM, it would be necessary to postulate that P388 cells effect an enormous concentration of the parent drug, if it were to be the actual cytotoxic species. For this reason, it is most likely that some metabolite of the title compound was the proximate inhibitor of IMP dehydrogenase. The delay in the inhibition of DNA synthesis (Fig. 6) also supports this notion. The 5′-monophosphate is the only metabolite identified to date with reasonable certainty. Although this nucleotide, on direct analysis, was found to be a comparatively potent non-competitive inhibitor of the enzyme versus IMP, with a K_i of 460 μM, it will remain for future studies to determine whether it is wholly responsible for the antimetabolic effects of the thiazole nucleoside. In this connection, it should be noted that the majority of the other known potent inhibitors of IMP dehydrogenase are also 5′-monophosphates.* These include 3-deazaguanylic acid, thiadiazole ribonucleoside 5′-monophosphate and ribavirin-5′-monophosphate whose K_i values versus the enzyme are 1.2, 0.1 and 0.25 μM respectively [10–14]. It is relevant that compound 8 in Fig. 2, which lacks the 5′-hydroxyl and so cannot be phosphorylated at that position, was wholly devoid of cytotoxicity. The inertness of the 2′-deoxythiazolenucleoside suggests either that it cannot be phosphorylated or, if phosphorylated, that it is precluded from interaction with its enzymic target by virtue of its lack of a 2′-hydroxyl group.

By analogy to certain other antipurines, it is possible that the thiazole nucleoside acted at more than one enzymatic site. It is relevant that ribavirin (an agent structurally and functionally related to the title compound) is thought to exert its antiviral effect via at least two mechanisms: inhibition of IMP dehydrogenase and perturbation of mRNA guanylyltransferase; in addition, ribavirin engenders an as yet unexplained expansion of the TTP pool [15]. Further studies will determine whether the thiazole nucleoside exhibits a similar diversity. Attention will also be focused on the biochemical effects of the title compound in representative murine tumors in order to pave the way for more rational clinical trials with this potent new oncolytic drug.

Acknowledgements—The authors wish to thank Ms. Beth Cohen, Ms. Antoinette Smith and Ms. Ellen McGee for their assistance in the preparation of this manuscript.

* The two other major classes of potent inhibitors of IMP dehydrogenase are complex phenolic compounds, of which mycophenolic acid is the prototype, and dinucleotide analogs of NAD, of which thiadiazoleadenine-dinucleotide is the prototype.

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