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STUDIES ON THE MECHANISM OF ACTION OF 2-β-D-RIBOFURANOSYLTHIAZOLE-4-CARBOXAMIDE (NSC 286193)—II

RELATIONSHIP BETWEEN DOSE LEVEL AND BIOCHEMICAL EFFECTS IN P388 LEUKEMIA *IN VIVO*

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Abstract—Administration of the novel thiazole C-nucleoside, 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC 286193), to BDF_1 mice bearing subcutaneous implants of P388 leukemia provoked a sharp depression in the concentration of intratumoral guanine nucleotides and a correspondingly large expansion of the IMP pools. Measurements of IMP dehydrogenase in the tumors of treated mice revealed that this enzyme was inhibited in a dose-responsive way, with ~50% inhibition engendered by the administration of the drug at a dose of 25 mg/kg and >90% inhibition by all doses >100 mg/kg. The inhibition of enzyme activity, seen after a dose of 250 mg/kg, reached a maximum 120 min after treatment and had subsided substantially 8 hr after dosing; by 24 hr, enzyme activity was fully restored. These results, coupled with the observation that the antitumor activity of the drug could be prevented in large part by the simultaneous administration of guanosine, support the conclusion that 2-β-D-ribofuranosylthiazole-4-carboxamide, after anabolism, exerts its antineoplastic effects via a state of guanine nucleotide depletion. In extracts of the tumors of mice given parenteral injections of the thiazole nucleoside, a potent dialyzable inhibitor of IMP dehydrogenase was demonstrable; its concentration fluctuated in parallel with enzyme inhibition. Although the chemical identity of the proximate inhibitory species has yet to be established, it is concluded on kinetic grounds that it is neither the native nucleoside nor its 5′-monophosphate.

2-β-D-Ribofuranosylthiazole-4-carboxamide (hereafter referred to as thiazole nucleoside or TR) is a novel C-nucleoside with notable antitumor activity against several murine leukemias and at least one important solid tumor, the Lewis lung carcinoma [1]. In a previous communication, we reported that this compound was capable of interrupting the biosynthesis of guanine nucleotides and so of nucleic acids in P388 cells in culture [2]. On the basis of available evidence it was concluded that the drug, or a metabolite of it, was arresting the generation of XMP (the immediate precursor of GMP) from IMP, by inhibiting the enzyme IMP dehydrogenase (IMPD). As a consequence of this inhibition, IMP pools were found to have expanded by as much as 15-fold. Further experiments have now been undertaken to determine whether these initial studies in cultured cells are of relevance to the action of the drug *in vivo*, using, as the test system, the subcutaneously transplanted murine leukemia P388, a neoplasm sensitive to the title compound. These experiments demonstrate that parenteral administration of the drug engenders powerful but transient inhibition of IMPD and provokes a state of guanine nucleotide depletion.

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MATERIALS AND METHODS

The reagents and techniques for the analysis of nucleoside phosphate pools by high performance liquid chromatography (HPLC) were the same as described in the first paper of the series [2]. [5-^{3}H]2-β-D-Ribofuranosylthiazole-4-carboxamide (1.96 Ci/mmole) was obtained from the Research Triangle Institute, Research Triangle Park, NC.

To study the influence of thiazole nucleoside on IMPD activity and nucleic acid biosynthesis, male BDF_1 mice, weighing 20–25 g, were injected s.c. with 1×10^6 cells/mouse of P388 leukemia. Seven days later, mice were treated i.p. with a dose of thiazole nucleoside (10–250 mg/kg) or saline for the duration specified in the legends to the appropriate tables and figures. Tumors were then excised and homogenized (1:10, w/v) in 0.1 M Tris–HCl buffer (pH 8.0) containing 20 mM KCl and 1 mM dithiothreitol, for the measurement of IMPD, or homogenized (1:4, w/v) in cold 5% perchloric acid (PCA), immediately neutralized with 40% KOH, centrifuged at 12,000 *g* for 3 min, and the supernatant fraction used for the measurement of the concentration of nucleotides.

Therapeutic studies

Groups of five BDF_1 mice (20–25 g) were injected i.p. with 1×10^6 cells/mouse of P388 leukemia in Hanks' balanced salt solution. Treatment (i.p.) was

started 24 hr later with thiazole nucleoside; doses and duration are specified in the table legends. Animals were fed Purina chow and water *ad lib.* Weights and mortalities were recorded daily.

For the studies related to the prevention or reversal of antitumor activity, groups of seven BDF_1 mice were injected i.p. with 1×10^6 cells/mouse. Treatment (i.p.) was instituted 24 hr later with saline, guanosine (500 mg/kg) injected as a suspension in 0.3% solution of hydroxylpropyl cellulose (Carter–Glogan Laboratories, Glendale, AZ), or nicotinamide (500 mg/kg) given simultaneously with thiazole riboside (100–400 mg/kg) or with saline daily for 5 days. Weights and mortalities were recorded daily.

IMPD activity

Reaction vessels were set up in triplicate with a fourth vessel serving as a control-blank. All vessels received 5 μl of 0.5 M KCl containing 8 mM uridine and 2.6 mM allopurinol at pH 8.0. In addition, control-blank vessels received 5 μl of 0.1 mM mycophenolic acid (an inhibitor of IMPD activity). These reactants were dried at room temperature overnight (16 hr). The drying step did not affect the stability of the constituents mentioned above. For the conduct of the assay, 5 μl of [2,8-^{3}H]IMP (375 μCi/ml, specific radioactivity 150 mCi/mmole) containing 1 mM NAD at pH 8.0, and 5 μl of cell extract as the source of IMPD were pipetted separately into the vessels. The reaction was started by centrifuging the reactants for 5 sec at 12,000 *g* followed by incubation at 37° for 15 min. The reaction was terminated by heating at 95° for 1 min. Vessels were then centrifuged for 1 min at 12,000 *g*, after which 5 μl of alkaline phosphatase from *Escherichia coli* (EC 3.1.3.1, 0.11 mg protein/ml, 30 units/mg protein, Sigma Chemical Co., St. Louis, MO) was added to each reaction vessel and incubated at room temperature for 20 min to hydrolyze IMP and XMP to their respective nucleosides. The vessels were centrifuged at 12,000 *g* for 1 min, and a 5 μl aliquot of the reaction mixture was spotted on Whatman 3 MM paper, overspotted with 5 μl of a mixture of inosine and xanthosine (10 mM each), and subjected to ascending chromatography at room temperature overnight (16 hr) using 80% (v/v) acetonitrile in water as the solvent. Spots corresponding to xanthosine were identified under u.v. light, excised, and eluted with 1 ml of water, and radioactivity was measured by scintillation spectrometry. Enzyme activities are expressed as nmoles of XMP formed per mg protein per hr.

Kinetic studies

Tumors were excised from BDF_1 mice bearing P388 leukemia transplanted s.c., and were homogenized (1:5, w/v) in 0.1 M Tris–HCl buffer (pH 8.0) containing 20 mM KCl and 1 mM dithiothreitol. The homogenate was centrifuged at 12,000 *g* for 3 min. To 3 ml of the supernatant fraction, 0.3 ml of 1 M sodium acetate (pH 5.0) was added, mixed, and left on ice for 5 min. The pellet containing IMPD was centrifuged at 12,000 *g* for 1 min and redissolved in one-third volume of the homogenizing buffer. The acid precipitation step yields a 10-fold increase in the specific activity of IMPD (*ca.* 60 nmoles of XMP synthesized per mg protein per hr) over the activity in the crude homogenate.

For kinetic studies, test vessels containing KCl, uridine and allopurinol were set up and allowed to dry overnight at room temperature as described above. Vessels were then divided into two groups. The first group received 5 μl of 1 mM NAD in 0.05 M Tris–HCl buffer (pH 8.0); 5 μl of [2,8-^{3}H]IMP (0.036 to 0.125 mM, specific radioactivity 1500 mCi/mmole); 5 μl of 2-β-D-ribofuranosylthiazole-4-carboxamide-5′-phosphate (2.0 to 4.0 mM) or water; and 5 μl of enzyme preparation. The second group received 5 μl of [2,8-^{3}H]IMP (0.063 mM, 0.375 mCi/ml, specific radioactivity 1500 mCi/mmole); 5 μl of NAD (2.0 to 8.0 mM) in 0.05 M Tris–HCl buffer (pH 8.0); 5 μl of 2-β-D-ribofuranosylthiazole-4-carboxamide-5′-phosphate (2.0 to 4.0 mM) or water; and 5 μl of enzyme preparation. Reactants were admixed by a 5-sec centrifugation at 12,000 *g* and incubated at 37° for 10 min. Reactions were terminated by heating at 95° for 1 min, then centrifuged for 1 min at 12,000 *g*, and further processed as detailed earlier.

Studies on the metabolism of thiazole nucleoside

Groups of three mice bearing s.c. P388 tumor were injected i.p. with 100 mg/kg thiazole nucleoside (containing 25 μCi/mouse of [5-^{3}H]2-β-D-ribofuranosylthiazole-4-carboxamide). Tumors were removed at the indicated time intervals, frozen, homogenized in 5% PCA and immediately neutralized. An aliquot of the neutralized extract was centrifuged at 12,000 *g* for 3 min, and the supernatant fraction was used for HPLC as described earlier [2]. The resulting pellet was used to study the incorporation of the drug into nucleic acids. For this purpose, the pellets were washed four times with 1 ml each of cold 5% PCA. To the pellets 100 μl of 40% KOH was added, digested at 95° for 30 min, cooled, and centrifuged at 12,000 *g* for 3 min. Aliquots were taken to determine the radioactivity by scintillation spectrometry and their absorbance at 260 nm.

RESULTS

Biochemical studies

Dose- and time-dependence of inhibition of IMPD activity in vivo. Inhibition of IMPD activity was demonstrable 2 hr following all doses of the thiazole nucleoside greater than 10 mg/kg and reached a maximum of 96% at the dose of 250 mg/kg (Table 1). It is noteworthy that all inhibition of IMPD was reversed by exhaustive dialysis of the extracts against the homogenizing medium, and that the inhibitor species could be recovered by lyophilization of the dialyzing buffer in titres that reflected the graduated doses of the drug given (Table 1).

When a therapeutic dose of 250 mg/kg of the drug was administered i.p., IMPD appeared to be strongly inhibited within 30 min; this effect reached an apparent maximum 2 hr after dosing (Table 2); thereafter, inhibition gradually decreased so that 24 hr after injection of the drug, IMPD in the extirpated tumors exhibited a normal specific activity. Once again, the titre of the dialyzable inhibitor fluctuated in parallel with the inhibition of IMPD.

Table 1. Dose–response of inhibition of IMP dehydrogenase by thiazole nucleoside *in vivo**

Dose (mg/kg)	Specific activity of IMP dehydrogenase [nmoles XMP formed · hr^{-1} · (mg protein)$^{-1}$]	% Inhibition of IMP dehydrogenase activity† Before dialysis	After dialysis	Titer of dialyzable inhibitor (dilution yielding 50% inhibition)
Saline	15.52 ± 0.34‡			
10	18.82 ± 7.34	<5	<5	
25	10.49 ± 4.16	33	<5	1:2
100	1.52 ± 0.24	90	<5	1:8
250	0.60 ± 0.21	96	<5	1:64

* Groups of five BDF_1 mice bearing s.c. P388 transplants were treated i.p. with saline or thiazole nucleoside. Two hours later, tumors were removed, homogenized (1:10, w/v) in 0.1 M Tris buffer (pH 8.0) containing 20 mM KCl 1 M dithiothreitol, and assayed for IMP dehydrogenase activity as detailed in Materials and Methods. One millilitre of supernatant fluid was also dialyzed in collodion dialysis thimbles against 50 ml of the homogenizing buffer. The outer bath was lyophilized, reconstituted with 1 ml of water, and clarified by centrifugation at 12,000 *g* for 3 min. The ability of serial halving dilutions of the resulting supernatant fraction to inhibit IMPD (a pH 5 enzyme from nodules of the P388 leukemia) was then examined under the conditions of analysis given above. Since extracts from saline controls were slightly inhibitory (25 ± 5%), all percentages of inhibition have been computed by comparison to control extracts prepared simultaneously.

† After drug exposure, the IMP concentration in tumors increased from ~25 to 125 μM. In the supernatant fractions used for assay, this IMP would be diluted to 2.5 to 12.5 μM, respectively, and in the reaction mixtures to ~1 and 4 μM respectively. Such exogenous IMP inevitably must have diluted the specific activity of the tritiated IMP used to measure IMPD and, so, have produced the appearance of inhibition. Because the contribution of this exogenous IMP is ~20% or less of the concentration of tritiated IMP employed, and because the increase was variable over time, no correction for this effect has been made.

‡ Mean ± S.D.

Time-dependence of inhibition by thiazole nucleoside of the incorporation of [8-^{14}C]*hypoxanthine into nucleic acids.* The incorporation of labeled hypoxanthine into acid-insoluble macromolecules was strongly inhibited 1 hr after administration of the thiazole nucleoside at a dose of 250 mg/kg. Inhibition reached a maximum 4 hr after dosing, was still profound at 8 hr, but had returned to nearly normal levels at the 24-hr sampling time (Table 3). Although there are quantitative differences between the per cent reductions of IMPD and of nucleic acid biosynthesis over time, especially in the 8-hr samples, the two sets of data (Tables 2 and 3) do establish that there is a definite lag to the inhibition of both

Table 2. Time-dependence of the inhibition of IMP dehydrogenase *in vivo* by thiazole nucleoside*

Time of sampling (min)	IMP dehydrogenase activity [nmoles XMP formed · hr^{-1} · (mg protein)$^{-1}$]	% Inhibition of IMP dehydrogenase activity compared to controls	Dilution yielding 50% inhibition
Control	8.80 ± 1.15†		ND‡
10	5.36 ± 1.14	39	ND
20	3.83 ± 0.77	57	ND
30	2.68 ± 0.76	70	ND
60	2.87 ± 1.90	67	1:16
120	0.49 ± 0.20	94	1:32
240	0.68 ± 0.06	92	1:32
480	5.56 ± 0.02	36	ND
1440	9.45 ± 1.06	<5	ND

* Groups of five BDF_1 mice bearing s.c. P388 tumor (7-day-old transplants) were treated i.p. with saline or 250 mg/kg thiazole nucleoside. Animals were killed at various time intervals, and tumor was removed and frozen on dry ice. After the last sampling, tumors were homogenized (1:10, w/v) in 0.1 M Tris buffer (pH 8.0) containing 20 mM KCl and 1 mM dithiothreitol. The homogenates were centrifuged at 12,000 *g* for 3 min and the IMP dehydrogenase activity was assayed in the supernatant fraction according to the technique detailed in Materials and Methods. The titer of dialyzable inhibitor was measured by the technique given in the legend to Fig. 1.

† Mean ± S.D.

‡ Inhibition not detectable.

Table 3. Time-dependence of the effect of thiazole nucleoside treatment on the incorporation of [8-^{14}C]hypoxanthine into nucleic acid *in vivo**

Time of sampling (hr)	Incorporation of hypoxanthine†		% of control
	Treatment		
	Saline	Drug	
1	40.2 ± 6.6	16.0 ± 3.9‡	40
2	33.4 ± 6.0	10.0 ± 1.6‡	30
4	32.6 ± 1.0	8.4 ± 2.0‡	26
8	31.6 ± 4.5	8.7 ± 2.7‡	28
24	31.4 ± 2.9	25.7 ± 6.4	82

* Groups of five male BDF_1 mice bearing s.c. P388 tumor 7–9 days after transplantation were injected i.p. with saline or 250 mg/kg thiazole nucleoside. Sixty minutes before sacrifice, the animals received allopurinol (50 mg/kg, i.p.) and 30 min before sacrifice they were injected i.p. with 5 μCi of [8-^{14}C]hypoxanthine. Tumors were then removed, frozen on dry ice, homogenized (1:4, w/v) in 5% PCA, and immediately neutralized with 40% KOH. The homogenate was centrifuged at 12,000 *g* for 3 min and the pellets were washed four times with 1 ml of 5% PCA. One milliliter of 1 N HCl was then added, and the pellet was suspended well, using a glass rod, heated for 95° for 10 min, and cooled. A 200-μl aliquot, after centrifugation, was taken for counting radioactivity and for measuring absorbance at 260 nm.

† Expressed as nCi of [8-^{14}C]hypoxanthine incorporated/A_{260} unit of depurinated nucleotide (mean ± S.D.)

‡ $P < 0.01$.

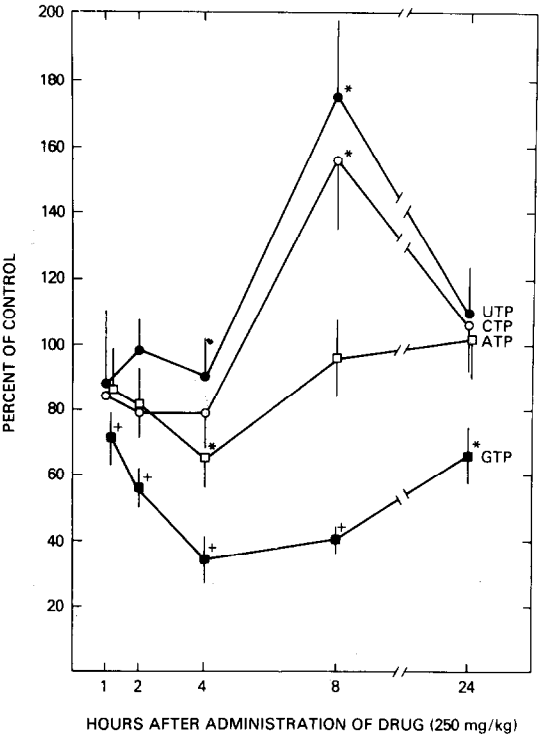

Fig. 1. Nucleoside triphosphate concentrations after administration of TR. Nucleoside triphosphate levels were measured in neutralized PCA extracts of P388 cells transplanted s.c. following administration of 250 mg/kg TR, i.p. Nucleotides were separated by HPLC using a Brownlee NH_2 precolumn (0.4 × 3 cm) coupled to a Brownlee NH_2 analytical column (0.4 × 10 cm) and eluting isocratically with 0.4 M KH_2PO_4 (pH 3.8):2% (v/v) acetonitrile at a flow rate of 4 ml/min. The range of control nucleotide concentrations (nmoles/g tissue) at the 1, 2, 4, 8 and 24 hr time intervals was: CTP, 83–230; UTP, 181–410; ATP, 451–859; and GTP, 178–394 for thirty-four separate determinations. An asterisk (*) denotes a statistically significant ($P < 0.05$) difference vs controls. A dagger (†) denotes a statistically significant ($P < 0.01$) difference vs controls.

processes and also that their rates of recovery are approximately equivalent.

Time-dependence of effects of thiazole nucleoside on nucleotide pools. Pursuant to the finding that the incoporation of hypoxanthine into nucleic acids was strongly inhibited by treatment with the thiazole nucleoside, its influence on nucleotide pools was next examined (Fig. 1). These studies indicated that the concentration of GTP underwent a pronounced and protracted reduction which was maximal 4 hr after treatment. Among the other nucleoside triphosphates which were measured, the only significant changes which occurred were the 35% decrease in ATP at 4 hr and the 160–180% increase in CTP and UTP at 8 hr after treatment. HPLC analysis of PCA extracts of tumor nodules from treated mice also revealed that the intratumoral concentration of IMP underwent a sizable expansion (450%) 2 hr after drug treatment (Fig. 2).

Time-dependence of intratumoral concentration of thiazole nucleoside and its 5′-monophosphate. The foregoing *in vivo* results are congruent with those obtained earlier *in vitro* [2] and support the concept that the thiazole nucleoside interrupts nucleic acid biosynthesis and cell growth by reversibly inhibiting IMPD. Unclear, however, is the nature of the molecular species actually responsible for this effect. As a first step toward the solution of this problem, mice bearing subcutaneous transplants of P388 leukemia were given an intraperitoneal injection of [5-^{3}H]thiazole nucleoside, and the rate of accumulation and/or metabolism of the drug was measured in these tumors by HPLC. As shown in Table 4, the concentrations of both thiazole nucleoside and its 5′-monophosphate were significantly lower at the 2-hr time point (i.e. at the time of maximal IMPD inhibition) that at either the 5-min or the 1-hr time points. This temporal relationship makes it unlikely that either of these molecules is proximately responsible for the inhibition of IMPD, a conclusion that is supported by kinetic data. For example, the K_i of the title compound for IMPD is 8.2 mM [2], yet the maximal concentration of drug measured in tumor was more than one hundred times lower than this, ~73 μM. Similarly, the K_i of the 5′-monophosphate of the thiazole nucleoside is 500 μM (Fig. 3), yet the maximal intratumoral level of this metabolite was over thirty times lower than this value. Since the carboxylic acid (fraction 2 of Table 4), is devoid of therapeutic activity [2], and fails to inhibit IMPD *in vitro* at 0.01 M (data not shown), it too can be discounted as the inhibitory species. By contrast, fraction 4 at 60 min (Table 4), which contains a

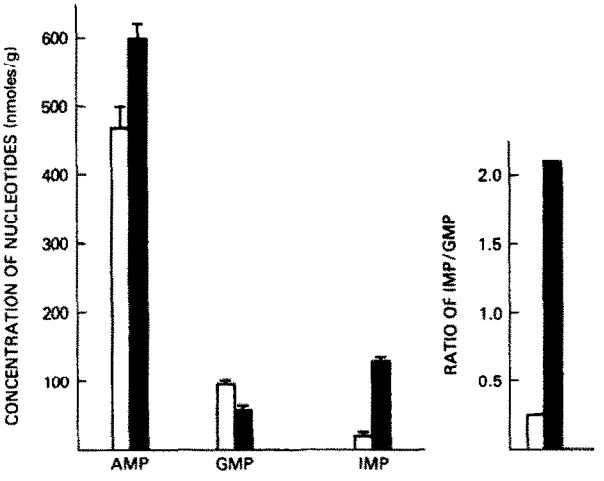

Fig. 2. Influence of thiazole nucleoside treatment on the concentration of purine nucleoside monophosphate. Groups of five mice bearing s.c. nodules of P388 leukemia were injected i.p. with 250 mg/kg TR or saline. Two hours later, tumors were excised, flash frozen on dry-ice and homogenized in 5% PCA, immediately neutralized; after centrifugation at 12,000 *g* for 3 min, an aliquot was used for the measurement of the concentrations of AMP, IMP and GMP by the HPLC technique detailed in Materials and Methods. Shaded bars indicate the concentrations in the treated group; open bars refer to the concentrations in the control group.

Table 4. Metabolism of [5-^{3}H]thiazole nucleoside by the P388 leukemia *in vivo**

Fraction no.	Concentration (nmoles of drug or metabolite/g tissue)		
	Time after administration of drug		
	5 min	60 min	120 min
1	53.9 (<5)	73.0 (<5)	13.1 (<5)
2	0.8 (<5)	1.8 (<5)	4.8 (<5)
3	14.0 (<5)	4.5 (<5)	0.2 (<5)
4	0.6 (<5)	3.5 (60)	1.5 (25)

* Groups of three mice bearing s.c. P388 tumors were injected i.p. with thiazole nucleoside (100 mg/kg, 25 μCi/mouse). At indicated time, the tumors and livers were removed and frozen. The tissues were then homogenized in 5% perchloric acid and immediately neutralized, and an aliquot was analyzed by HPLC as detailed in Materials and Methods; fractions were counted by liquid scintillation spectrometry. To measure the IMPD-inhibitory activity of these HPLC fractions, they were diluted 1:10 (v/v) with 0.01 M Tris–HCl (pH 7.6) (final dilution 1:200); 5-μl aliquots of the resulting solutions were tested for their ability to inhibit a partially purified preparation of IMPD from the P388 leukemia using the methodology presented in the text. Figures in parentheses indicate per cent inhibition of IMPD measured under the conditions described above. Fraction 1: parent drug; fraction 2: 2-β-D-ribofuranosylthiazole-4-carboxylic acid; fraction 3: 2-β-D-ribofuranosylthiazole-4-carboxamide-5′-phosphate; and fraction 4: cumulative ^{3}H-radioactivity eluting after the 5′-monophosphate.

number of unresolved and unidentified acidic metabolites, did inhibit 60% of the IMPD activity *in vitro* at a final dilution of 1:200. Attempts are underway to identify the molecule or molecules responsible for this effect.

Since the foregoing results suggested that the drug was presumably anabolized to higher phosphates the possible incorporation of [5-^{3}H]thiazole nucleoside into nucleic acids was determined. BDF$_1$ mice bearing s.c. transplants of P388 were injected i.p. with NSC 286193 (100 mg/kg; 25 μCi/mouse of [5-^{3}H]drug) for 6, 30 or 120 min. Tumors and livers were removed, flash frozen on dry ice and homogenized (1:4) in 5% PCA. One milliliter of the homogenate was transferred into separate tubes and centrifuged. The pellets were washed four times with 5% PCA. One hundred microliters of 40% KOH was added to the pellet, digested at 95° for 30 min, cooled, and centrifuged at 12,000 *g* for 3 min. Aliquots were taken to determine the radioactivity by scintillation spectrometry and absorption at 260 nm. The amount of ^{3}H-radioactivity isolated with the nucleic acids from tumors and livers was extremely small (corresponding to <0.002% and <0.01% substitution respectively), and the identity of this radioactivity was therefore not pursued further.

Therapeutic studies

In view of the reduction in guanosine nucleotide

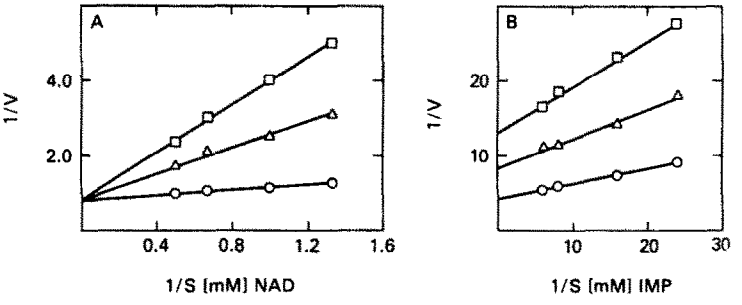

Fig. 3. Kinetics of inhibition of IMP dehydrogenase activity by 2-β-D-ribofuranosylthiazole-4-carboxamide-5′-phosphate (TMP). The methodology used for these studies is detailed in Materials and Methods. Panel A refers to the study with NAD as the variable substrate and panel B refers to the study with IMP as the variable substrate. Symbols: (○) refers to the assay in the absence of TMP; (△ and □) refer to 0.5 and 1 mM TMP (final concentration) respectively.

Table 5. Influence of nicotinamide, guanosine and/or thiazole nucleoside treatment on the survival of mice bearing P388 leukemia*

Regimen	Treatment	Dose (mg/kg)	Survival period (mean days ± S.D.)	% T/C
Q1D × 1	Saline		10.8 ± 1.4	
	TR	25	10.8 ± 1.4	100
		100	12.1 ± 1.1	112
		250	12.5 ± 1.2	116
		500	12.3 ± 2.0	114
Q1D × 5	Saline		10.8 ± 1.4	
	TR	100	14.7 ± 0.7†	131
		200	17.3 ± 2.7†	160
		400	20.5 ± 1.9†	190
Q1D × 5	Saline		10.8 ± 1.4	
	TR	200	17.3 ± 2.7†	160
	Nicotinamide	500	10.0 ± 2.8	100
	TR	200	17.6 ± 2.0†	168
	+ Nicotinamide	500		
	Guanosine	500	11.4 ± 1.0	105
	TR	100	13.6 ± 3.2	126
	+ Guanosine	500		
	TR	200	13.1 ± 1.7†	121
	+ Guanosine	500		
	TR	400	13.8 ± 0.7†	128
	+ Guanosine	500		

* Groups of seven male BDF_1 mice weighing 20–25 g were injected i.p. with 10^6 cells of P388 tumor. Twenty-four hours later, treatment was started by i.p. injection of mice with thiazole nucleoside or saline and/or nicotinamide, guanosine or saline for 1 or 5 days.
† $P < 0.01$.

pool sizes noted on treatment with thiazole nucleoside, the effect of guanosine on the therapeutic effect of the drug was examined. Thiazole nucleoside alone produced significant increases in the life-span of mice bearing P388 leukemia when injected as five daily doses, but not as a single dose (Table 5). Concurrent daily administration of guanosine largely cancelled the therapeutic effects seen. Nicotinamide was not able to counter the antineoplastic activity of the drug (Table 5).

DISCUSSION

Relationship between dose level of thiazole nucleoside and effects on nucleotide pools

The present series of experiments with the P388 leukemia growing *in vivo* demonstrate that marked expansions of the IMP pools and marked contractions of the GTP pools were detected following administration of therapeutic levels of the drug. The contention that inhibition of IMPD is responsible for these changes is supported by both the time–course and dose–response experiments, which, in concert, establish that this, a key step in purine nucleotide biosynthesis [3], is inhibited very strongly after intraperitoneal injections of thiazole nucleoside. The possibility exists that expansion of the IMP pool is amplified by factors in addition to direct inhibition of IMPD by thiazole nucleoside or its anabolities. For example, the decrease in GTP pools arising as a consequence of IMPD inhibition may reinforce or supplement the effects to be seen from direct inhibition of IMPD, since GTP is known to be a potent inhibitor of adenylate deaminase from murine tumors [4]. Lowering of the guanine nucleotide pools could thus permit the deaminase to degrade AMP to IMP at an accelerated rate and, thus, contribute to the accumulation of the latter nucleotide. In this connection, it is worthwhile noting that three other prototypical inhibitors of IMPD, 2-amino-1,3,4-thiadiazole, mycophenolic acid and ribavirin, also produce alterations of nucleotide pools closely comparable to those produced by thiazole nucleoside, i.e. marked depressions in guanine nucleotides and elevation of one or more pyrimidine nucleotides [5, 6].

The rate of onset of biochemical changes following a therapeutic dose of thiazole nucleoside was rather rapid, e.g. IMPD inhibition was maximal at 2 hr, and inhibition of GTP pool sizes and of the incorporation of hypoxanthine into nucleic acids was maximal at 4 hr. Recovery was correspondingly rapid, with some effects of the drug no longer detectable at 8 hr and complete recovery by 24 hr. The therapeutic schedule for thiazole nucleoside most frequently utilized to date has been daily administration of the drug for 5 or 9 days [1]; the present results showing rapid reversibility of its biochemical effects would indicate that a more sustained and possibly greater therapeutic effect could be obtained by more frequent administration, e.g. q 8 hr or q 12 hr.

Biochemical consequences of the inhibition of IMPD

Consequent on the inhibition of IMPD, two important biochemical results ensued: accumulation of IMP and restricted availability of XMP and thence of guanine nucleotides.

With respect to the first of these effects, IMP accumulation, it has been suggested that this nucleotide can, for example, impair the fidelity of DNA transcription by inhibiting one or more of the proofreading mechanisms [7]. The almost complete reversal of the therapeutic effects of thiazole nucleoside by the administration of guanosine, however, would appear to imply that the depletion of guanine nucleotides, rather than the accumulation of hypoxanthine nucleotides, is primarily responsible for the action of the drug. It is likely, however, that IMP could contribute to the toxicity, if not to the therapeutic efficacy of the drug, since the accumulation of the latter nucleotide should in turn result in increased production of inosine and hypoxanthine and, thus, (in man and other primates) of uric acid, with the attendant complications of hyperuricemia and hyperuricosuria, as is seen with the antitumor drug 2-amino-1,3,4-thiadiazole [8].

With respect to the second biochemical consequence stemming from inhibition of IMPD, it is likely that the resultant restricted availability of XMP leads to depletion of GTP, and so of dGTP.* In this connection, it is relevant that inhibition by mycophenolic acid of IMPD in mouse lymphoma cells deficient in hypoxanthine guanine phosphoribosyl transferase has been shown to result in cytotoxicity and inhibition of DNA synthesis reminiscent of that reported here. Although exogenous deoxyguanosine restores dGTP pools in these malignant lymphocytes, this restoration is *not* accompanied by a resumption of DNA synthesis; conversely, expansion of both the GTP and dGTP pools *does* permit this process to resume. On the basis of such evidence, it is has been suggested that normal concentrations of GTP, as well as dGTP, are required for the synthesis of DNA [9]. It is possible that the P388 leukemia cells used in the present study exhibit an analogous dual requirement. Indeed, the ability of exogenous guanosine to partially counteract the oncolytic actions of thiazole nucleoside (Table 5) very likely reflects an expansion of first the GTP and then the dGTP pools.

Depletion of GTP would also be expected to have severe consequences on RNA metabolism. Apart from reducing substrate availability, GTP depletion may perturb 5'-"cap" formation in both mRNA [10] and small nuclear RNA species [11] and, hence, interfere with the normal functioning and processing of mRNA. In addition, protracted depressions of GTP would be expected to have effects on protein synthesis (for which this nucleotide is an essential requirement), on the assembly of certain complex lipids (for which GDP-mannose is needed), on microtubule polymerization, and on the synthesis of cyclic GMP.

These studies define a temporal relationship between the administration of thiazole nucleoside and the changes in purine and pyrimidine nucleotide levels consequent on IMPD inhibition. A question, however, arises: if IMPD inhibition is primarily responsible for the therapeutic action of the drug, why do other IMPD inhibitors (e.g. 2-amino-1,3,4-thiadiazole) not show comparable striking and unusual activity against relatively refractory tumors such as Lewis Lung and L1210? A possible explanation would appear to be that the IMPD-inhibitor species generated from thiazole nucleoside is more active than that generated from other agents of the thiazole class. The present evidence indicates that this inhibitory species is not the 5'-monophosphate, and other anabolites with IMPD-inhibitory activity are presently being sought. Another possible explanation would be different pharmacokinetic characteristics of thiazole nucloside, e.g. the transport characteristics of the compound could result in higher concentrations in tumor and lower concentrations in sensitive host tissues such as bone marrow than is the case with other agents of this class. This possibility is also being investigated.

* Direct measurement of deoxyribonucleotides in 60% methanolic extracts of subcutaneously grown P388 tumor nodules have established that dGTP pools are depressed by 36, 36, 67, 64 and 33% at 2, 4, 8, 24 and 48 hr after a single intraperitoneal dose of TR (200 mg/kg). This finding establishes that the drug restricts the availability of guanine nucleotides for both RNA and DNA synthesis (unpublished observations).

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