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STUDIES ON THE MECHANISM OF ACTION OF 2-β-D-RIBOFURANOSYLTHIAZOLE-4-CARBOXAMIDE—V

FACTORS GOVERNING THE RESPONSE OF MURINE TUMORS TO TIAZOFURIN

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Abstract—The pharmacological effects and metabolism of tiazofurin have been compared in the six transplantable tumors comprising the NCI rodent tumor panel, viz. the P388 leukemia (S); the L1210 leukemia (S); the Lewis lung carcinoma (S); the B16 melanoma (R); the colon 38 carcinoma (R); and the M5076 sarcoma (R), where (S) denotes sensitivity and (R) resistance to tiazofurin. In addition, a variant of the P388 leukemia rendered resistant to the drug *in vitro*, and maintaining stable resistance *in vivo*, P388/TR, was also studied. Intraperitoneal administration of tiazofurin (100 mg/kg) resulted in a 3- to 30-fold greater accumulation of thiazole-4-carboxamide adenine dinucleotide (TAD), the proposed active metabolite of the drug in S versus R lines. In general, levels of TAD, percent inhibition of IMP dehydrogenase (mean 40% in S versus 10% in R), depression in the concentration of guanosine nucleotides, (50% in S versus 20% in R) and percent elevation of levels of IMP (500% in S versus 60% in R) correlated well with sensitivity or resistance. However, the B16 melanoma, although resistant to tiazofurin treatment, showed certain biochemical features characteristic of an S line. The sensitive and resistant tumors displayed comparable abilities to phosphorylate tiazofurin, but there was significant depression only in the R lines of the pyrophosphorylase which converts tiazofurin-5'-monophosphate to TAD (mean 78 nmoles/mg protein/hr in S versus 22 nmoles/mg protein/hr in R). The naturally resistant tumors were also found to exhibit a greater ability to degrade synthetic TAD than the sensitive lines (mean 102 nmoles/mg protein/hr in R versus 29 nmoles/mg protein/hr in S lines). The state of sensitivity or resistance could not be attributed to the basal levels of IMP dehydrogenase, to the specific activities of the enzymes of purine salvage, or to the basal concentration of purine and pyrimidine nucleotides. Moreover, treatment with tiazofurin did not influence the enzymes of TAD synthesis or of purine salvage.

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, TR) exhibits potent oncolytic activity against several murine leukemias and is curative of the Lewis lung carcinoma [1]. Consequent to tiazofurin treatment, guanosine nucleotide pools fall precipitously in treated cells [2, 3]. On the basis of kinetic measurements, it was concluded that a metabolite of the parent compound, but not the drug itself, was reponsible for the antitumor activity of tiazofurin [2]. Not only has this metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD), been isolated from treated tumors, but it has also been synthesized by both chemical and enzymatic means [4–6] and proven to be a potent inhibitor of IMP dehydrogenase activity of the P388 leukemia [2, 4, 5] and Chinese hamster ovary cells in culture [7]. Anabolism of tiazofurin to TAD was also observed in human lymphoid tumor cell lines [8] and human lung cancer lines in culture [9].

Confirmation that tiazofurin exerted its oncolytic effects as a consequence of its anabolism to TAD was provided by studies with a variant of the P388 leukemia (P388/TR) rendered resistant to tiazofurin [3]. In this variant line, little or no accumulation of TAD was demonstrable. Moreover, exposure of these refractory cells to drug resulted in no depression of guanosine nucleotide pools. A similar mechanism of resistance has also been reported in CHO cells [7]. In both of these cases, the resistance appeared to be due to a block in the synthesis of TAD [3, 7]. Since the two variants in question were developed by the use of tremendous selection pressures, it was felt to be important to examine the activity of tiazofurin in tumors naturally sensitive or resistant to treatment with the drug. Towards this end, we have measured the anabolism of tiazofurin, along with its pharmacological effects, in the six neoplasms comprising the rodent tumor panel of the National Cancer Institute, National Institutes of Health. Against two of these lines (leukemias L1210 and P388), tiazofurin exhibits significant, but not curative, activity with T/C values of ~230%; against the intravenously inoculated Lewis lung carcinoma, the drug is curative, producing a high percentage of long-term, tumor-free survivors over the dosage

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range of 12.5 to 800 mg/kg given intraperitoneally for the first 9 days after implantation of the tumor [1].

In contrast to these three susceptible tumors, tiazofurin is inactive against the B16 melanoma, colon 38 carcinoma, M5076 sarcoma, and the variant of the P388 leukemia with acquired resistance to tiazofurin (P388/TR). Pursuant to the rationale explained earlier, we have therefore conducted a comprehensive examination of factors governing the therapeutic activity or inactivity of tiazofurin in these transplanatable rodent tumors.

MATERIALS AND METHODS

[5-^{3}H]Tiazofurin (1.96 Ci/mmole) was obtained from the Research Triangle Institute, Research Triangle Park, NC. [2-^{3}H]Hypoxanthine (4.9 Ci/mmole), for the synthesis of labeled IMP, [8-^{14}C]hypoxanthine (52.8 mCi/mmole) and [8-^{14}C]adenine (62.0 mCi/mmole) were purchased from Amersham/Searle, Arlington Heights, IL. Yeast alcohol dehydrogenase (sp. act. 300 units/mg; 30 mg/ml) was the product of the Boehringer Mannheim Corp., Elmsford, NY. Tiazofurin (NSC 286193) was obtained from the Drug Synthesis and Chemistry Branch, NCI, NIH. Chemically synthesized TAD was a gift from Dr. G. Gebeyehu, DCT, NCI, NIH. All other chemicals were of the highest purity available.

Evaluation of antitumor activity, in vivo

Antitumor evaluations *in vivo* were conducted by the screening program of the Drug Evaluation Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI). Mice were obtained through the Animal Genetics and Production Branch, DTP, DCT, NCI, and were kept in holding rooms until their weights were appropriate (minimum 17 and 18 g for females and males respectively) for tumor transplantation and experimentation. Animals were allowed food (fat content 6–12%) and water *ad lib*. Tumor lines were obtained from the frozen tumor bank of the Developmental Therapeutics Program. Protocols employed for screening with the intraperitoneally implanted B16 melanoma, L1210 lymphoid leukemia and P388 lymphocytic leukemia have been published previously [10]. The experimental methodology for testing materials against the carcinogen-induced transplantable colon 38 carcinoma have been described elsewhere [11], as have the characteristics of the intravenously implanted Lewis lung carcinoma [12] and the M5076 sarcoma [13–15]. The subline of P388 leukemia resistant to tiazofurin (P388/TR) was not obtained from the frozen tumor bank but was established as reported earlier [3]. For evaluation of the sensitivity of the tumors to tiazofurin, CDF_1 mice were implanted intraperitoneally with either 1×10^6 P388, 1×10^6 P388/TR or 1×10^5 L1210 leukemia ascites cells; BDF_1 mice were inoculated with either 0.5 ml of a 1:10 B16 melanoma brei intraperitoneally, 70 mg of a colon 38 carcinoma fragment subcutaneously in the axillary region, or 0.5 ml of a 1:10 M5076 sarcoma brei subcutaneously; $B_6C_3F_1$ mice were inoculated intravenously with 1×10^6 Lewis lung carcinoma cells. Treatment with tiazofurin as a solution in water or saline was initiated 24 hr (48 hr for the colon 38 carcinoma) post tumor implantation on day 0 according to the schedules listed in Table 1, and a series of logarithmically spaced dosages were tested. Each dosage level was administered to ten mice (six for P388 and P388/TR), and thirty to forty control mice received only the vehicle used to administer the drug. For survival assays, T/C percent was computed as median survival time of treated mice × 100/survival time of controls. The percent increase in life span (ILS) is equal to the T/C percent − 100. For tumor growth inhibition assays, T/C percent was computed as median weight of treated tumors × 100/median weight of control tumors. The percent tumor weight inhibition is 100 − T/C percent. Tumor size was measured across two diameters, and weight was computed assuming nearly ellipsoid shape and specific gravity not significantly different from 1.0.

For enzymologic and metabolic studies, male BDF_1 mice weighing 20–25 g were subcutaneously transplanated with tumor cells (10^5–10^6 cells/mouse) or tumor brei as indicated earlier. For basic enzymologic studies tumors were excised 7–9 days later in the case of leukemias or 9–15 days in the case of remaining tumors.

Metabolism of TR

Male BDF_1 mice bearing 7- to 15-day-old implants of various tumors were injected intraperitoneally with TR, 100 mg/kg (specific radioactivity: 10 μCi/mg of drug). Two hours following treatment, animals were killed and tumors were excised and immediately flash-frozen between blocks of dry ice. Specimens were then homogenized in ice-cold 10% trichloroacetic acid (TCA) (1:5, w/v). Homogenates were centrifuged for 2 min at 12,000 *g*, and supernatant fractions were removed and neutralized with 0.5 M trioctylamine in freon [16]. Neutralized samples were loaded onto a Partisil 10-SAX column (Waters Associates, Milford, MA), preequilibrated with 0.03 M ammonium phosphate buffer [4]; under these conditions, tiazofurin (TR), tiazofurin 5′-monophosphate (TR-5′-MP) and TAD eluted at 3, 7, and 18 min respectively. By this high performance liquid chromatography (HPLC) method, purine and pyrimidine nucleotides were also quantitated before and after treatment with tiazofurin.

Enzyme analysis

Sample preparation. Mice bearing subcutaneous tumors were killed by cervical dislocation; tumors were promptly removed and washed briefly with homogenization buffer consisting of 0.25 M sucrose, 1 mM dithiothreitol (DTT) and 20 mM Tris–HCl, pH 7.4. Homogenizations were carried out in 4 vol. of the above buffer using a glass–Teflon homogenizer. Homogenates were passed through four layers of cheesecloth to remove cell debris and adipose tissue. The filtrate thus obtained was centrifuged at 3000 *g* for 10 min in a refrigerated Sorvall centrifuge, and supernatant fractions were further centrifuged in an Eppendorf centrifuge at 12,000 *g* for 2 min at 4°. Enzyme extracts (12,000 *g* supernatant fractions) were used for the enzymatic analysis

of IMP dehydrogenase (IMPD), TR kinase, hypoxanthine/guanine phosphoribosyl transferase (HPRTase) and adenine phosphoribosyl transferase (APRTase). The pellet (3000 *g*) was washed twice with the homogenization buffer and finally resuspended in 20 mM Tris–HCl, pH 7.4, containing 1 mM DTT. This suspension was used as a source of enzyme for the measurement of NAD pyrophosphorylase activity. The whole homogenate was filtered through two layers of cheesecloth and was used for determining TADase activity.

Enzymatic synthesis of TR-5′-MP (TR-kinase). Ability to phosphorylate tiazofurin to its 5′-monophosphate (TR-5′-MP) was determined using 12,000 *g* supernatant fraction as the enzyme source. The assay mixture in a total volume of 10 μl contained: 0.5 mM [5-^{3}H]tiazofurin (sp. act. 86.0 mCi/mmole), 0.01 M ATP–$MgCl_2$ or 0.01 M $MgCl_2$ and 0.05 M Tris–HCl, pH 8.0. ATP-free vessels served as assay blanks. To initiate the reaction, 5 μl of the enzyme extract was dispensed under the lid of the Eppendorf vessel, and the reactants were admixed by brief centrifugation. Following a 20-min incubation at 37°, the reaction was terminated by heating at 95° for 1 min. After centrifugation, 5-μl aliquots of the supernatant fraction were spotted on Whatman 3 MM chromatographic paper, and overspotted with 5 μl of 0.01 M tiazofurin and TR-5′-MP. Chromatograms were developed overnight in the ascending mode using ethanol–1 M ammonium acetate, pH 7.0 (70:30, v/v) as solvent system. TR-5′-MP spots were excised, eluted with water, and analyzed for radioactivity by scintillation spectroscopy. Under these conditions, tiazofurin and TR-5′-MP exhibited R_f values of 0.77 and 0.32 respectively.

Measurement of NAD pyrophosphorylase activity. The activity of this enzyme was measured by a modification of the method of Pinder *et al.* [17]. The assay mixture contained 3.0 mM ATP (pH 7.6), 0.2 M nicotinamide, 25 mM glycylglycine buffer with 7.5 mM $MgCl_2$, in the presence or absence of 8.0 mM nicotinamide mononucleotide (NMN), in a total volume of 0.5 ml. The reaction was initiated with 0.2 ml of homogeneously suspended pellet. Following a 20-min incubation at 37°, the reaction was terminated by heating in a boiling water bath for 1 min. Assay vessels were centrifuged at 12,000 *g* for 2 min, and the supernatant fractions (450 μl) were transferred to spectrophotometric cuvettes containing 100 mM sodium-pyrophosphate buffer, pH 8.6, with 100 mM semicarbazide, 30 mM glycine and 230 mM ethanol in a total volume of 0.7 ml. After initial recording of the absorbance at 340 nm, alcohol dehydrogenase (ADH) was added to each cuvette to a final concentration of 26 μg/ml, and 30 min later a final reading at 340 nm was obtained. The difference in absorbance was used for quantitation of NAD. For computation of results, endogenous NAD (measured using an assay mixture lacking NMN) was subtracted from total NAD concentrations. Pyrophosphorylase activity was found to be linear with time up to 30 min and with various protein concentrations (0.25 to 1.20 mg).

Degradation of TAD (TADase activity). Tumors were homogenized in 4 vol. (w/v) of buffer containing 0.03 M Tris–HCl with 0.015 M $MgCl_2$, pH 7.6. The ability of whole tumor homogenates to degrade TAD was measured using chemically synthesized TAD which was >95% pure as determined by the HPLC system described earlier [6]. In the conduct of the assay, 50 μl of 2.0 mM TAD was incubated with 100 μl of tumor homogenate. Following a 15-min incubation at 37°, the reaction was terminated by adding 50 μl of 30% TCA. Control vessels received TAD immediately after TCA treatment. Following centrifugation at 12,000 *g* for 2 min, supernatant fractions were separated, neutralized, and analyzed on HPLC as detailed earlier. TADase activity was determined by measuring the decrease in the amount of exogenously added TAD, or the corresponding increase in AMP and TR-5′-MP concentrations, compared to controls.

IMP dehydrogenase assay. Radiolabeled substrate for the assay, [2-^{3}H]IMP, was prepared from [2-^{3}H]hypoxanthine as described previously [2]. For the enzyme assay, 5-μl aliquots of 0.5 M KCl with 2×10^{-5} M allopurinol were dried at the apexes of Eppendorf centrifuge vessels; 5 μl of the substrate mixture containing 2.86×10^{-4} M [2-^{3}H]IMP (200 μCi/ml) and 3 mM NAD was added to each vessel. The reaction was initiated with a 5-μl aliquot of the enzyme extract. All other assay conditions were as described previously [18].

Hypoxanthine and adenine phosphoribosyl transferases. These purine salvage enzyme activities were measured by a minor modification of the method described previously [19]. The assay mixture for the measurement of hypoxanthine phosphoribosyl transferase contained: 5 μl of [8-^{14}C]hypoxanthine (sp. act. 55 mCi/mmole, 50 μCi/ml) and 0.01 M 5-phosphoribosyl-1-pyrophosphate (PRPP)–$MgCl_2$ in 0.1 M Tris–HCl, pH 8.0, or buffer alone. The reaction was initiated with 5 μl of the enzyme extract. After a 5-min incubation at 37°, the reaction was terminated with 10 μl of 2 N HCl. An aliquot (10 μl) of the 12,000 *g* supernatant fraction was spotted on Whatman 3MM paper, overspotted with 5 μl of IMP plus hypoxanthine (10 mM each), and developed by ascending chromatography for 16 hr using ethanol–1 M ammonium acetate, pH 7.0 (70:30, v/v) as a solvent. Radioactivity was measured in the IMP spots.

For the measurement of adenine phosphoribosyl transferase, [^{14}C]hypoxanthine was replaced by [8-^{14}C]adenine (sp. act. 58 mCi/mmole, 50 μCi/ml). Enzyme activity was calculated according to the radioactivity present in the AMP spots.

Protein determination

Protein was determined by the method of Bradford [20] using the Bio-Rad Kit; 5- to 10-μl aliquots of suitably diluted samples were mixed with 1.0 ml of the Bio-Rad Reagent, and absorption at 595 nm was measured within 5–60 min. Bovine serum albumin, Fr 5 (5–15 μg), was used as standard.

RESULTS

Sensitivity of murine tumors to tiazofurin, in vivo

The therapeutic effectiveness of tiazofurin against a number of murine transplanted tumors is illustrated in Table 1. The drug was very effective against the

Table 1. Response of murine transplanatable tumors to tiazofurin*

Tumor system	Treatment schedule (i.p.)	FED†	Control mice		Test mice			
			Median survival time (days)	Median tumor weight (mg)	Opt. dose (mg/kg/inj)	Increased life span (%)	Tumor inhibition (%)	FED survivors/ Total
Sensitive								
P388 leukemia, i.p.	qd, days 1–9	30	11.0		700	145		0/6
	qd, days 1–5	30	10.6		600	77		0/6
Lewis lung carcinoma, i.v.	qd, days 1–9	60	25.0		200			10/10
L1210 leukemia, i.p.	qd, days 1–9	60	8.4		512	134		0/10
Resistant								
B16 melanoma, i.p.	qd, days 1–9	60	19.1		800	−1		0/10
Colon 38 carcinoma, s.c.	qd, days 2,9	20		736	1600		12	0/10
M5076 sarcoma, s.c.	qd, days 1–9	17		750	300		9	0/10
P388/TR leukemia, i.p.	qd, days 1–5	30	9.0		600	1		0/6

* Groups of six or ten CDF_1, BDF_1 or $B_6C_3F_1$ mice were inoculated with tumor on day 0 as indicated in Materials and Methods. Treatment with logarithmically spaced doses of tiazofurin dissolved in water or saline was initiated 24 or 48 hr later according to the schedules listed in the table. The data obtained with the most effective dose (optimum dose) for the sensitive tumors, or the highest non-lethal dose tested for the non-responsive tumors, are shown. Experiments in which tiazofurin demonstrated activity were confirmed at least once.

† Final evaluation day.

intravenously implanted Lewis lung carcinoma producing 100% (10/10) 60-day survivors following intraperitoneal administration of a 200 mg/kg dose on days 1–9 and a high percentage of long-term, tumor-free survivors over a wide dosage range (12.5 to 800 mg/kg/dose). Good activity also was observed against the intraperitoneally implanted P388 and L1210 leukemias. Maximum increased life spans of 145% at 700 mg/kg in the P388 system and 134% at 512 mg/kg in the L1210 system were obtained following intraperitoneal administration on days 1–9. Lower dosage levels also demonstrated good antitumor effects (ILS ≥ 50%). All data obtained with these three sensitive tumors systems were confirmed in at least one other experiment. In contrast, the B16 melanoma, colon 38 carcinoma and the M5076 sarcoma were shown to be naturally resistant to tiazofurin under the experimental conditions evaluated. In addition, the variant of the P388 leukemia (P388/TR) rendered resistant to the drug *in vitro* showed no response toward the drug *in vivo* over the tested dosage range of 150–800 mg/kg. In the same experiment, tiazofurin at a dose of 600 mg/kg administered on days 1–5 increased the life span of mice bearing the P388 sensitive line by 77%.

Metabolism of tiazofurin in murine tumors

Two hours after treatment with tiazofurin (100 mg/kg, 10 μCi/mg of drug), the metabolites of the drug formed by subcutaneous tumors were analyzed on HPLC (Table 2). Markedly variable concentrations of the parent drug were achieved in the various tumors studied (~22 nmoles/g in colon 38 to 196 nmoles/g in P388/TR), but these levels did not correlate with the sensitive or resistant nature of the lines. Similar results were obtained for the accumulation of tiazofurin-5′-monophosphate, where the observed levels showed no correlation either with the concentration of its precursor (tiazofurin) or with the sensitivity or resistance of these tumors. By contrast, TAD accumulation showed marked differences in the two groups. Thus, a 3- to 30-fold higher concentration of TAD was observed in the sensitive as compared to the resistant tumors. The highest accumulation of TAD was observed in the Lewis lung carcinoma (19 nmoles TAD/g) followed by L1210 and P388/S. Among the naturally resistant lines, B16 showed the highest concentration (1.3 nmoles/g) of the active anabolite, a value which is, nevertheless, markedly lower than that measured in any of the sensitive lines. No TAD could be demonstrated in the extracts of the P388/TR tumor line.

Enzymes involved in the synthesis and degradation of TAD

Pursuant to the finding that there is evidence of impaired accumulation of TAD in the resistant tumors, the enzymology involved in the regulation of TAD concentration was studied in both groups of tumors. TAD synthesis from tiazofurin is a two-step process. The first enzymatic step is the conversion of tiazofurin to its 5′-monophosphate (TR-5′-MP). The kinase responsible for this phosphorylation has not yet been identified. Nevertheless, tumor extracts were assayed for their ability to phosphorylate [5-^{3}H]tiazofurin. Variable kinase activity was observed in the tumors studied. However, this enzyme activity did not correlate with the state of sensitivity or resistance (Table 3). The critical step resulting in TAD accumulation and sensitivity is believed to be the conversion of TR-5′-MP to TAD. NAD pyrophosphorylase is known to catalyze this reaction with NMN or TR-5′-MP as substrate [4]. NAD-pyrophosphorylase activity was found to be significantly higher in the sensitive as compared to the resistant tumors (Table 3). In the sensitive lines, the enzyme activity varied from ~90 nmoles/hr/mg protein in L1210 to ~70 nmoles/hr/mg protein in Lewis lung. Among the resistant tumors B16 showed the highest enzyme activity (~47 nmoles/hr/mg protein). In general, the concentrations of TAD measured *in vivo* (Table 2) correlated well with the NAD pyrophosphorylase activity measured *in vitro* (Table 3).

In vivo TAD accumulation will depend on both the rate of its synthesis and the rate of its breakdown. Unlike NAD-pyrophosphorylase activity, the TAD degradative rate (TADase activity) was found to be significantly higher in the naturally resistant group (67–140 nmoles TAD degraded/hr/mg protein) compared to the sensitive tumor lines (24–37 nmoles

Table 2. Sensitivity and metabolism of tiazofurin in murine tumors*

Tumor	Sensitivity to tiazofurin	Concentration of tiazofurin and its metabolites (nmoles/g)		
		Tiazofurin	TR-5′-MP	TAD
P388/S	S	152 ± 80	8.6 ± 3.6	3.9 ± 0.6
Lewis lung	S	52 ± 22	1.5 ± 0.4	19.0 ± 3.9
L1210	S	72 ± 19	3.6 ± 1.0	11.7 ± 2.0
B16	R	34 ± 2	3.5 ± 0.4	1.3 ± 0.2
Colon 38	R	22 ± 41	2.3 ± 0.3	0.9 ± 0.1
M5076	R	83 ± 20	2.5 ± 0.3	0.6 ± 0.1
P388/TR	R	196 ± 118	3.1 ± 0.5	<0.05

* Groups of five male BDF_1 mice bearing s.c. nodules of tumors were injected intraperitoneally with the drug, 100 mg/kg (10 μCi [5-^{3}H]tiazofurin/mg). Two hours later, mice were killed, and tumors were excised and immediately flash-frozen on dry-ice. Metabolites were extracted with 10% TCA treatment followed by neutralization of supernatant fractions with 0.5 M trioctylamine in freon. Neutralized samples were analyzed on HPLC as detailed in Materials and Methods. Values are means ± S.D.

Table 3. TAD biosynthetic and degradative enzyme activities in murine tumors*

Tumor	Sensitivity to tiazofurin	Specific activity† (nmoles/hr/mg protein) TR-kinase	NAD-pyrophosphorylase	TADase	NAD-pyrophosphorylase/TADase
P388/S	S	2.6 ± 0.6	75.0 ± 5.0	24.0 ± 6.7	3.13
Lewis lung	S	2.9 ± 1.0	70.0 ± 8.0	37.0 ± 18.0	1.90
L1210	S	0.4 ± 0.1	90.0 ± 8.0	25.0 ± 6.0	3.60
B16	R	1.0 ± 0.2	47.0 ± 2.0	100.0 ± 19.0	0.47
Colon 38	R	0.4 ± 0.1	37.0 ± 0.8	140.0 ± 25.0	0.26
M5076	R	0.9 ± 0.2	17.0 ± 3.3	67.0 ± 14.0	0.25
P388/TR	R	2.4 ± 0.4	12.6 ± 0.9	26.0 ± 3.7	0.46

* Groups of five male BDF_1 mice bearing subcutaneous tumors were killed by cervical dislocation. Tumors were removed and homogenized in ice-cold homogenization buffer. Extracts for TR kinase (12,000 *g* supernatant fraction), NAD-pyrophosphorylase (3000 *g* pellet) and TADase (tumor homogenates) were prepared and assayed for enzyme activities as described in Materials and Methods. Specific activity for TR kinase or NAD pyrophosphorylase is represented as nmoles of TR-5′-MP or NAD formed/hr/mg protein respectively. Specific activity for TADase is represented as nmoles of exogenously added synthetic TAD degraded/hr/mg protein.

† Values are expressed as mean ± S.D.

TAD degraded/hr/mg protein). The ratios of NAD pyrophosphorylase to TADase was found to be in the range of 0.25 to 0.47 in the resistant tumors whereas a significantly higher ratio (1.9 to 3.6) was observed in the sensitive group.

Influence of tiazofurin treatment on IMP dehydrogenase activity in vivo

It has been shown previously that TAD is a potent inhibitor of IMP dehydrogenase [4]. Two hours after tiazofurin treatment (100 mg/kg), IMPD activity was measured in the sensitive and resistant tumors (Table 4). The basal activity (saline-treated controls) of IMPD varied from one tumor to another, but no correlation was observed with the sensitivity or resistance of these tumors to tiazofurin.

After treatment with the drug, the highest percent inhibition (64%) of IMPD was observed in the Lewis lung carcinoma, followed by leukemia L1210 (29%) and P388/S (23%). The resistant group (P388/TR, colon 38 and M5076) showed minimal inhibition (8–11%), with the exception of the B16 melanoma, a line whose IMPD was inhibited up to 25% following tiazofurin treatment.

Influence of tiazofurin treatment on the concentrations of nucleotides

The pool sizes of endogenous purine and pyrimidine nucleotides of the seven tumors under study are shown in Table 5. These pools were generally comparable in the sensitive and resistant tumors. The triphosphates of adenosine and guanosine were higher than the mono- and diphosphates in all lines except for colon 38; in this connection it is relevant to point out that the alkaline phosphatase and 5′-nucleotidase activities were found to be similar among all lines (results not shown). Changes in the nucleotide levels after tiazofurin treatment are presented in Table 6. Greater than 50% reduction in guanosine nucleotide concentration was observed in the sensitive lines, compared to a 0–43% decrease in the resistant lines. Decreases in guanosine nucleotide pools were accompanied by an increase of IMP concentrations in all lines except the colon 38. Once again, the drug-resistant B16 melanoma showed certain biochemical features characteristic of a sensitive line: a 43% decrease of guanosine nucleotide pools, accompanied by a significant increase in IMP levels following an exposure to tiazofurin. A variable effect

Table 4. Influence of treatment with tiazofurin on IMP dehydrogenase activity in murine tumors*

Tumor	Sensitivity to tiazofurin	IMPD activity (nmoles of XMP formed/mg protein/hr) Saline control	Tiazofurin-treated
P388/S	S	3.6 ± 0.7	2.8 ± 0.4
Lewis lung	S	3.3 ± 0.9	1.2 ± 0.6
L1210	S	0.7 ± 0.1	0.5 ± 0.2
B16	R	0.8 ± 0.1	0.6 ± 0.1
Colon 38	R	1.2 ± 0.3	1.0 ± 0.2
M5076	R	1.8 ± 0.4	1.6 ± 0.3
P388/TR	R	0.9 ± 0.2	0.8 ± 0.1

* Groups of five male BDF_1 mice bearing subcutaneous tumors were treated with either saline or 100 mg/kg tiazofurin, given i.p. 2 hr before killing. Tumors were then excised and extracts were assayed for IMPD activity as described in Materials and Methods. Values are means ±S.D.

Table 5. Concentrations of nucleotides in tumors sensitive and resistant to tiazofurin*

Tumor	Sensitivity to tiazofurin	AMP (4.0)	ADP (23.5)	ATP (33.9)	GMP (10.5)	GDP (26.0)	GTP (37.1)	IMP (8.1)	CTP +UTP (31.0)	UMP +TMP (5.9)
P388/S	S	518	630	958	41	138	248	16	131	67
		±116	±105	±247	±13	±27	±52	±5	±35	±16
Lewis lung	S	663	718	914	62	132	177	26	139	62
		±144	±221	±537	±7	±31	±76	±7	±116	±10
L1210	S	432	594	959	41	150	237	21	265	58
		±133	±201	±256	±9	±63	±76	±6	±66	±20
B16	R	711	607	1613	33	125	296	14	410	75
		±3	±25	±137	±1	±9	±20	±4	±48	±10
Colon 38	R	1794	1976	456	135	384	96	22	26	201
		±323	±349	±102	±45	±48	±24	±9	±4	±46
M5076	R	727	729	1965	40	175	412	8	472	93
		±106	±95	±139	±10	±28	±30	±3	±33	±12
P388/TR	R	433	650	1074	35	123	245	24	394	87
		±95	±131	±102	±12	±24	±30	±9	±55	±27

* Concentrations of nucleotides are expressed as nmoles (±S.D.)/g of tumor tissue. Groups of five male BDF_1 mice bearing subcutaneously implanted tumors were killed by cervical dislocation, and excised tumors were immediately flash-frozen between blocks of dry-ice. TCA extracts (10%) were prepared and analyzed on HPLC as described in Materials and Methods. Values in parentheses represent the elution time of standard nucleotides under these experimental conditions.

on pyrimidine nucleotide pools (generally an increase) was observed in the tumor lines after drug treatment.

Purine salvage enzyme activities

Measurements of purine salvage enzymes were undertaken next, for the purpose of uncovering any differences in the abilities of the seven lines to reutilize preformed purines. The activities of HGPRTase and APRTase in the murine tumors studied are shown in Table 7. Although a wide range of specific activities of these enzymes (45 nmoles/hr/mg protein to 1378 nmoles/hr/mg protein for HPRTase and 64 nmoles/hr/protein to 549 nmoles/hr/mg protein for APRTase) was observed, there was no consistent correlation with sensitivity or resistance. Treatment with tiazofurin for 2 hr before extraction of the tumors did not influence the salvage enzymology in any of the tumors studied (results not shown).

DISCUSSION

The principal purpose of the present study was to determine what factors contribute to the activity or inactivity of tiazofurin in tumors naturally sensitive or resistant to the drug. The tumors of the NCI rodent tumor panel were felt to be well suited for this purpose since their responsiveness to tiazofurin has been carefully examined, and their utility as predictors for clinical activity is well established [21]; moreover, three of these tumors were sensitive and three resistant to the drug, a distribution lending balance to the study.

Among the variables measured, several showed distinct correlations with the sensitivity and resistance of the tumors. These will be discussed in turn.

Enzymatic transformation of tiazofurin to TAD

Two hours after a single intraperitoneal dose of tiazofurin of 100 mg/kg, the three sensitive lines

Table 6. Influence of treatment with tiazofurin on the concentrations of nucleotides in murine tumors sensitive or resistant to the drug*

Tumor	Sensitivity to tiazofurin	% of Saline control				
		Adenosine nucleotides	Guanosine nucleotides	IMP	UMP +TMP	CTP +UTP
P388/S	S	100	49	737	138	329
Lewis lung	S	69	45	413	100	162
L1210	S	69	39	365	144	40
B16	R	91	57	766	123	164
Colon 38	R	84	69	94	82	107
M5076	R	75	68	188	77	66
P388/TR	R	100	100	100	71	127

* Groups of five male BDF_1 mice bearing subcutaneous tumors were treated with either saline or 100 mg tiazofurin/kg. Two hours later tumors were excised and immediately flash-frozen. Nucleotide extraction and analysis are detailed in Materials and Methods.

Table 7. Activity of purine salvage enzymes in murine tumors sensitive or resistant to tiazofurin*

Tumor	Sensitivity to tiazofurin	Enzyme activity (nmoles/hr/mg protein)	
		HPRTase	APRTase
P388/S	S	87.0 ± 11.0	94.0 ± 13.0
Lewis lung	S	45.0 ± 5.0	91.0 ± 46.0
L1210	S	340.0 ± 20.0	476.0 ± 12.0
B16	R	108.0 ± 17.0	64.0 ± 4.0
Colon 38	R	292.0 ± 32.0	335.0 ± 24.0
M5076	R	1378.0 ± 206.0	549.0 ± 45.0
P388/TR	R	84.0 ± 8.0	125.0 ± 37.0

* Subcutaneous tumors from groups of five male BDF_1 mice were removed and immediately homogenized. The 12,000 *g* supernatant fraction from the homogenates was assayed for HPRTase and APRTase activity, using [8-^{14}C]hypoxanthine or [8-^{14}C]adenine as substrates respectively. Details of this assay are described in Materials and Methods. Values are expressed as means ± S.D.

accumulated between 3 and 30 times as much TAD as their resistant counterparts. Moreover, the enzyme believed to catalyze the synthesis of TAD (NAD pyrophosphorylase) exhibited a mean specific activity in the S lines that was ~3 times that of the R lines.

Although B16 melanoma and colon 38 carcinoma showed NAD pyrophosphorylase activity which was ~50% of the sensitive group (Table 3), the TAD concentrations achieved in these tumors *in vivo* were disproportionately depressed (Table 2). This could be explained by the fact that these naturally resistant lines exhibited TADase activity which was 3- to 6-fold higher than that seen in the sensitive group. The ratio of synthetic to degradative activity was substantially greater than unity (>1.9) for sensitive tumors but substantially less than unity (<0.5) for resistant tumors. This greater TAD-degradative capacity of R lines, along with their relatively low NAD pyrophosphorylase activity, would result in a state of low TAD accumulation and thus resistance to the drug. Viewed together, these results indicate that a critical concentration of TAD (>3.5 μM; Table 2) must be maintained in a given tumor if a cytocidal response to tiazofurin is to be achieved.

While the present experiments document that naturally resistant tumors accumulate significantly less TAD than their naturally sensitive counterparts, they do stand in contrast to the results with artificially-resistant lines, which, it will be recalled, contained virtually none of the dinucleotide after dosing with tiazofurin. This absolute absence of TAD biosynthesis explains, of course, why such lines can tolerate cultivation in medium containing the drug at concentrations as high as 10 mM [3].

Effect on the target enzyme, IMP dehydrogenase

The principal consequence of the *in vivo* presence of TAD at or above its critical level is inhibition of IMP dehydrogenase. As Table 4 documents, the extent of inhibition of this target enzyme *in vivo* was generally greatest in the sensitive tumors; nevertheless, even in these lines inhibition was far from total, the maximum value achieved being 64% in the extremely sensitive Lewis lung carcinoma. Since inhibition of IMPD by TAD is freely and fully reversible [5], these modest effects are doubtless a consequence of inevitable dilution of the inhibitor in the course of extraction and assay of the enzyme. In the light of these considerations, then, it should be stressed that the inhibitions presented in Table 4 are apparent and so not strictly representative of those prevailing *in vivo*.

Perturbation of nucleotide concentrations

Parenteral administration of tiazofurin produced three major types of perturbation of nucleotide pools.

The first change, presumably a direct consequence of the inhibition of IMPD, was an expansion of the pool size of IMP. This effect was most prominent in the sensitive neoplasms, reaching, for example 737, 413 and 365% of control in the P388/S, Lewis lung carcinoma and L1210 leukemia respectively. However, even in the B16 melanoma, a tumor refractory to tiazofurin treatment, a notable expansion of the IMP levels was measured. Since this tumor forms more TAD than the other two resistant lines studied, the observed expansion of its IMP pools may reflect greater inhibition of IMP dehydrogenase *in situ*, than that observed *in vitro*, for reasons discussed earlier.

A second major effect of tiazofurin on nucleotide concentration was ~50–60% reduction in guanosine nucleotides seen in the three sensitive tumors versus ~0–40% reduction in the resistant tumors. The modest nature of these reductions even in the sensitive lines merits comment inasmuch as a halving of GTP pools would appear to be inadequate to produce a lethal disruption of the recovery of the target cells. The single dose of tiazofurin used in these studies was deliberately set at 100 mg/kg in order to unmask differences in the effects of the drug on the S and R lines without, at the same time "swamping" the target enzyme IMPD, which is susceptible to inhibition both by the parent drug and its monophosphate. Since a single dose of this magnitude is without therapeutic effect, it is warranted to speculate that a 50% reduction in G pools is, in fact, a sublethal event.

The third major action of tiazofurin was to enlarge

the pyrimidine nucleotide levels. Since this effect was most prominent in the sensitive lines, it is likely to somehow be a consequence of the impediment in guanosine nucleotide biosynthesis documented earlier to be most prominent in these lines. One possible explanation for this paradoxical increase might be that it results from a failure to utilize pyrimidine nucleotides for nucleic acid synthesis when that process is arrested as a consequence of an unavailability of guanosine nucleotides.

Ancillary contributing factors

An assessment was also made of a variety of other factors which might influence the activity of tiazofurin. These include the endowment of the enzymes of purine salvage in the seven lines and the basal specific activity of IMPD in extracts of untreated cells. Neither of these variables correlated in an unambiguous way with the state of sensitivity or resistance. It should be pointed out, however, that one of the resistant lines (M5076) did exhibit an unusually high level of hypoxanthine/guanine phosphoribosyl transferase. This line manifestly was well equipped to cope with a state of guanosine nucleotide deprivation. In view of its exquisite sensitivity to tiazofurin, it is also relevant that the Lewis lung carcinoma exhibited the lowest capacity for salvaging preformed guanine.

From these studies, we conclude that TAD accumulation *in vivo* is the principal and most important factor in determining natural sensitivity or resistance to tiazofurin, and that accumulation of this dinucleotide is regulated by a balance between the synthetic activity (NAD pyrophosphorylase) and the degradative rate (TADase activity).

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