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STUDIES ON THE MECHANISM OF ACTION OF TIAZOFURIN (2-β-D-RIBOFURANOSYLTHIAZOLE-4-CARBOXAMIDE) VI

BIOCHEMICAL AND PHARMACOLOGICAL STUDIES ON THE DEGRADATION OF THIAZOLE-4-CARBOXAMIDE ADENINE DINUCLEOTIDE (TAD)

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Abstract—In order to exert its antitumor effects, the C-nucleoside tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) is converted to the dinucleotide TAD (thiazole-4-carboxamide adenine dinucleotide), an inhibitor of IMP dehydrogenase (IMPD). With few exceptions, sensitive tumors (such as the P388 leukemia) have been found to accumulate substantially more of this inhibitory dinucleotide than resistant strains (exemplified by the colon 38 carcinoma). Previous studies have attributed this difference to a depressed capacity to synthesize TAD on the part of tumors refractory to tiazofurin. In the present study, a second contributory factor has been identified, viz. an enhanced ability to degrade preformed TAD. This degradation has been traced to a soluble phosphodiesterase present at high levels in tumors naturally resistant to tiazofurin. Using standard techniques, this TAD-phosphodiesterase has been purified 200-fold from the colon 38 carcinoma. The activity so purified readily hydrolyzed TAD and ADP-ribose, but exhibited a comparatively weak activity toward NAD and thymidine-5′-monophosphate-nitrophenyl ester. ADP-Ribose was also an excellent inhibitor of the hydrolysis of TAD. It is concluded, on the basis of these results, that TAD-phosphodiesterase plays an important role in the expression of the oncolytic activity of tiazofurin. The suggestion is also made that ADP-ribose may be the natural substrate for this enzyme.

In susceptible cells, the oncolytic nucleoside tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) is anabolized to a dinucleotide TAD‡ (thiazole-4-carboxamide adenine dinucleotide), which powerfully inhibits IMP dehydrogenase (IMPD) and so restricts the production of guanosine phosphates needed for nucleic acid biosynthesis [1–10]. This restriction ultimately leads to cell death. Previous studies from our laboratory [8, 10] have established two factors which render any given cell or tissue sensitive to tiazofurin: a rich endowment with NAD pyrophosphorylase (the enzyme which forms TAD), and a comparatively low capacity for hydrolyzing TAD, an activity attributable to a cellular phosphodiesterase (hereafter called TAD-phosphodiesterase). In an effort to gain further information on this degradative capacity, we have more recently carried out experiments whose aim was to partially purify and characterize TAD-phosphodiesterase from a suitable source and to examine its interaction with various endogenous and synthetic substrates and inhibitors with the ultimate goal of prolonging the half-life of TAD *in vivo*. The present paper recapitulates the results of these experiments.

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‡ Abbreviations: TAD, thiazole-4-carboxamide adenine dinucleotide; NHXD, nicotinamide hypoxanthine dinucleotide; NAAD, nicotinic acid adenine dinucleotide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; ADP-ribose, adenosine diphosphate ribose; GDP-mannose, guanosine diphosphate mannose; CDP-choline, cytidine diphosphate choline; UDP-glucuronic acid, uridine diphosphate glucuronic acid; TRMP, thiazole-4-carboxamide-5′-monophosphate; TMPN, thymidine-5′-monophosphate-nitrophenyl ester; HPLC, high pressure liquid chromatography; TCA, trichloroacetic acid; IMPD, inosinic acid monophosphate dehydrogenase; DTT, dithiothreitol; NMN, nicotinamide mononucleotide; NAMN, nicotinic acid mononucleotide; NAD, nicotinamide adenine dinucleotide; NADH, NAD (reduced form); NADP, nicotinamide adenine dinucleotide phosphate; NADPH, NADP (reduced form); A[5′]P-P[5′]A: P^1,P^2-di(adenosine-5′)pyrophosphate; A[2′]P[5′]A: adenylyl(2′→5′)-adenosine; A[2′]P[5′]G: adenylyl(2′→5′)guanosine; A[3′]P[5′]G: adenylyl(3′→5′)guanosine.

MATERIALS AND METHODS

Materials

[5-^{3}H]Tiazofurin (1.96 Ci/mmole) was obtained from the Research Triangle Institute, Research Triangle Park, NC; [8-^{14}C]ATP (52 mCi/mmole) for the synthesis of labeled TAD was purchased from Amersham/Searle, Arlington Heights, IL; tiazofurin (NSC 286193) was obtained from the Drug Synthesis

and Chemistry Branch, NCI, NIH. Venom phosphodiesterase (*Crotalus durissus*) and hog liver NAD pyrophosphorylase were obtained from the Boehringer Mannheim Corp., Elmsford, NY. DEAE Sephadex (A-50) was purchased from Pharmacia (Piscataway, NJ). Unlabeled nucleotides (natural as well as synthetic) were obtained from the Sigma Chemical Co., St. Louis, MO. TAD was synthesized as described previously [11]. All other reagents used were of the highest purity available.

In vivo *metabolism of tiazofurin*

For metabolic studies, male BDF_1 mice weighing 25–30 g were inoculated subcutaneously with 10^6 P388 cells or with tumor fragments of the colon 38 carcinoma as described previously [8]. Tumors were allowed to grow subcutaneously for 6–7 days in the case of the P388 leukemia, and for 15–21 days in the case of the colon 38 carcinoma. Tumor-bearing animals were injected i.p. with tritiated tiazofurin, 200 mg/kg, 50 μCi/mouse. At specific intervals after this treatment, groups of four animals were killed, and tumors were promptly removed and flash-frozen on dry-ice. Sample preparation and HPLC analysis were essentially the same as described previously [1]; 100 μl of neutralized sample was loaded onto a prestandardized Partisil 10-SAX column (Waters Associates, Milford, MA) and eluted with an ammonium phosphate gradient for the determination of drug metabolites and nucleotide pools. TAD was quantitated from the amount of radiolabel in the TAD peak and the specific activity of the tiazofurin injected. Radioactivity was determined by scintillation counting in a Beckman model LS-7900 counter. Nucleotide levels in the sample were determined by comparison to the area under the curve of standard reference nucleotides, using a Waters Data Module (Waters Associates).

Purification of TAD-phosphodiesterase

Colon 38 tumors were grown subcutaneously in male BDF_1 mice as described earlier. After a period of 15–21 days, tumors were excised and placed in ice-cold saline solution. After the removal of adhering fat, tumors were washed thoroughly with saline, their capsules were removed, and the central tumor mass was pooled for homogenization in 4 vol. of 30 mM Tris–HCl, pH 7.5, 1 mM DTT and 10 mM $MgCl_2$, using a Polytron homogenizer (Brinkmann, Westbury, NY). The homogenate was passed through four layers of cheesecloth to remove cell debris and centrifuged at 105,000 *g* for 1 hr in an ultracentrifuge (Beckman, model L2-65B) using a Type 40 rotor. To the resultant supernatant fraction, solid ammonium sulfate was added with constant stirring to 50% of saturation. Precipitated proteins containing the enzyme were recovered by centrifugation at 10,000 *g* for 20 min in a Sorvall centrifuge. The pellet so obtained was redissolved in the homogenization buffer and dialyzed overnight against two changes of the same buffer. The dialysate was loaded onto a DEAE-Sephadex A-50 column (20 cm × 1.65 cm) pre-equilibrated with 30 mM Tris–HCl, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.05 M NaCl. Elution was carried out with a gradient of 0.05 M to 0.5 M NaCl in buffer at a flow rate of 0.5 ml/min. A total of 90 fractions (3 ml each) was collected and analyzed for protein content as well as for enzyme activities. Protein concentration was determined by the method of Bradford [12] using a Bio-Rad kit. Enzyme activities of alkaline phosphodiesterase and TAD-phosphodiesterase were determined as described below.

Alkaline phosphodiesterase assay

Alkaline phosphodiesterase activity was determined spectrophotometrically [13]. To 500 μl of the substrate, consisting of 3 mM thymidine-5′-monophosphate-nitrophenyl ester, 3 mM $MgCl_2$ and 0.05 M Tris–HCl, pH 8.4, was added 100 μl of the eluted fraction. After 1 hr at 25°, 500 μl of 0.01 M NaOH was added and the absorbance was monitored at 420 nm. Blanks received elution buffer in lieu of the enzyme.

TAD-Phosphodiesterase assay

TAD-Phosphodiesterase activity was monitored by a radiometric method as described previously [10]. [^{14}C]TAD was synthesized using hog liver NAD-pyrophosphorylase, [^{14}C]ATP and tiazofurin-5′-monophosphate (TRMP). Conditions for the synthesis and further purification of the labeled TAD by reverse phase HPLC have been described previously [10]. A 25-μl aliquot of the eluted fraction was incubated with [^{14}C]TAD at a final concentration of 5 mM, in 20 mM Tris–HCl, pH 7.6, and 15 mM $MgCl_2$ in a total volume of 35 μl. After 30 min of incubation at 37°, the reaction was stopped by heating at 95° for 1 min. The radiolabeled [^{14}C]AMP resulting from the degradation of [^{14}C]TAD was isolated on a miniature column of Dowex 1-X8 formate and was taken as a direct measure of TAD degradative activity.

Fractions containing the highest TAD-phosphodiesterase activity and minimal contamination by alkaline phosphodiesterase activity (Fractions 38–41) were pooled and extensively dialyzed against three changes of 100 vol. of buffer containing 0.03 M Tris, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT and 1 mM $CaCl_2$. The dialyzed enzyme was concentrated by further dialysis against Carbowax *PEG* 20,000.

Determination of TAD-Phosphodiesterase activity by HPLC

TAD-Phosphodiesterase activity was measured using either TAD or its congeners as substrate. Assay mixtures contained 5 μl of 30 mM TAD or its congeners, 5 μl of the buffer (0.06 M Tris, 0.03 M $MgCl_2$, pH 7.6) and 10 μl (7.5 μg protein) of the concentrated enzyme. Following a 30-min incubation at 37°, the reaction was terminated by the addition of 250 μl of 10% TCA. Control vessels received the respective dinucleotide after the TCA treatment. Following centrifugation at 12,000 *g* for 2 min, supernatant fractions were separated, neutralized, and analyzed on anion exchange HPLC as described earlier [8]. Since phosphodiesteratic cleavage of these dinucleotides generates an equimolar concentration of both of the nucleotide components, enzyme activity could be determined by measuring either the decrease in the amount of added substrate or the increase in the corresponding products. Since the reaction was car-

Table 1. HPLC assay of phosphodiesterase products

Chemical agent	Elution time (min)	Product 1	Elution time (min)	Product 2	Elution time (min)
TAD	17.1	AMP	3.9	TRMP	6.3
ADP-Ribose	17.2	AMP	3.9	Ribose 5′-MP	ND*
FAD	21.4	AMP	3.9	FMN	8.2
GDP-Mannose	19.2	GMP	10.3	Mannose-1-P	ND
UDP-Glucuronate	22.9	UMP	5.7	Glucuronate-1-P	ND
CDP-Choline	5.0	CMP	2.9	Choline-2-P	ND
NAAD	16.8	AMP	3.9	NAMP	8.0
NHXD	6.9	NMN	2.5	IMP	8.0
NADPH†	24.9; 26.1	2′,5′-ADP	20.8	NMN (reduced)	2.3
NADH†	17.2; 18.4	AMP	4.1	NMN (reduced)	2.3
NADP	20.7	2′,5′-ADP		NMN	2.4
NAD‡	18.6	AMP‡	16.1	NMN‡	8.6
A[5′]P-P[5′]A	15.7	AMP	4.0	AMP	4.0
A[2′]P[5′]A	2.8	AMP	4.0	Adenosine	1.9
A[2′]P[5′]G	3.4	GMP	9.9	Adenosine	1.8
A[3′]P[5′]G	3.5	3′-AMP	4.7	Guanosine	1.9

Chemical agents listed in the table were analyzed on HPLC before and after treatment with either TAD-phosphodiesterase or snake venom phosphodiesterase. The separations shown were carried out on a radial compression column of Partisil-10 SAX developed at a flow rate of 1 ml/min, with 0.03 M ammonium phosphate, pH 3.5, isocratic for 10 min, followed by a linear gradient over 20 min to 0.6 M ammonium phosphate, pH 4.4. Before tho next sample, the column was equilibrated for 10 min with initial buffer. HPLC purity of the listed chemical agents, monitored at a 254 nm wavelength, was greater than 95%. Enzyme assay details are given in Materials and Methods.

* Not determined.

† Two major peaks were present in all commercially available preparations of NADPH and NADH tested.

‡ NAD analysis was performed by reverse phase HPLC (cf. Materials and Methods).

ried out for a sufficiently short time that substrate hydrolysis never exceeded 15%, decrease in substrate was found to be a poor measure of enzyme activity. However, measurement of the generation of either product was found to be a reproducible index of enzyme activity. For dinucleotides having AMP as one of their components, quantitation of this nucleotide on HPLC was taken as a measure of dinucleotide degradation. For dinucleotides lacking AMP as one of their components, other products were quantitated. This HPLC methodology was sufficient to separate all the products from their corresponding substrates upon enzyme treatment, as listed in Table 1.

Reverse phase HPLC for the measurement of NAD degradation

Phosphodiesteratic degradation of NAD was analyzed by means of a Radial Pak C-18 column (Waters Associates) using a Beckman HPLC system. Elution was carried out using a linear gradient between solvent A (0.03 M ammonium acetate, pH 6.05) and solvent B (25% methanol) at a flow rate of 2 ml/min for 25 min. The retention times of NAD, NMN and AMP were 18.66, 8.65 and 16.15 min, respectively, under these conditions.

Snake venom phosphodiesterase activity

Venom phosphodiesterase activity was determined towards TAD and its analogs in essentially the same manner as described above for TAD-phosphodiesterase. Assay mixtures contained 5 μl of 30 mM TAD or its congeners, 5 μl of the buffer (0.06 M Tris–HCl), 0.03 M $MgCl_2$, pH 7.6, and 10 μl (0.33 μg protein) of the commercial venom enzyme.

RESULTS

Time-course of tiazofurin metabolism and nucleotide pool changes in P388 lymphocytes versus colon 38 carcinoma in vivo

As shown in Fig. 1, the accumulation of TAD was found to be markedly different in two representative transplantable tumor lines. The first, P388 leukemia, is known to be responsive to tiazofurin; the second, colon 38 carcinoma, is known to be resistant to the drug. TAD reached its highest concentration at 1 hr after parenteral administration of tiazofurin in the case of colon 38, and at 2 hr in the P388 tumor. However, whereas the concentration of TAD fell to a biologically insignificant level within 6 hr in the colon 38 tumor, P388 lymphoblasts maintained a relatively much higher concentration of this anabolite at least up to 8 hr, with detectable levels persisting up to 24 hr after treatment. Computer-assisted integration of the areas under the curves (AUC) of TAD levels (Fig. 1) yielded values of 86.57 nmoles · hr · (g tumor)$^{-1}$ and 9.55 nmoles · hr · (g tumor)$^{-1}$ for the P388 and colon 38 tumors respectively. The enzymologic bases for this marked difference forms the subject of this report.

As was discussed in our previous report [8], the nucleotide pools most influenced by TAD are those derived from guanosine and inosine. Alteration in these pools correlated well with the time–course of accumulation of TAD in the colon 38 carcinoma

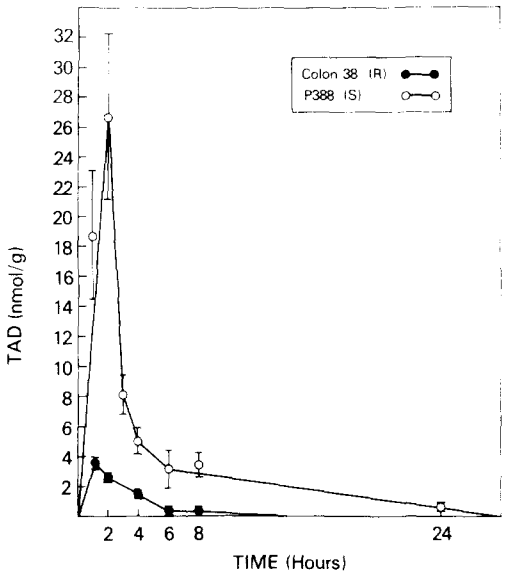

Fig. 1. Tiazofurin levels in murine tumors. Male BDF_1 mice bearing either P388 leukemia or colon 38 carcinoma were injected with radiolabeled tiazofurin (200 mg/kg, 50 μCi/mouse). At indicated intervals tumors were removed and analyzed for tiazofurin metabolic products, as described in Materials and Methods. Each data point represents the mean of four animals ± S.D.

(Table 2). In the first 2 hr after treatment, maximal depression of guanosine nucleotide levels was achieved, adenosine nucleotides remained essentially unchanged (not shown) and, as expected, IMP pools underwent a marked expansion. All of these alterations returned to normal within 4–6 hr of initial treatment. On the other hand, in the P388 lymphosarcoma, the perturbation of nucleotide levels was considerably more protracted; and although the accumulation of TAD peaked at 2 hr after dosing, its maximal impact on guanosine nucleotide levels was not observed until 4–6 hr after treatment with tiazofurin. In the first 2 hr after dosing, guanosine mono- and triphosphate levels were reduced by approximately 50% and GDP pools underwent an approximately 25% reduction. Further decline in these nucleotides continued, reaching nadir levels at 4 hr. Maximum inhibition was observed in GMP pools (approximately 90%); at the same time, GDP and GTP pools were reduced by 60 and 70% respectively. IMP reached its maximum 2 hr after the initial treatment and remained at an elevated level (600–800% of control) up to at least 8 hr. Adenosine nucleotides remained essentially unchanged throughout (results not shown). All altered nucleotide pools had essentially returned to normal by 24 hr after treatment.

Characterization of TAD-Phosphodiesterase

As was reported previously [8], tumors resistant to tiazofurin contained a significantly higher level of an enzyme activity capable of cleaving TAD at the phosphodiester linkage. To explore the pharmacological relevance of this activity, we embarked on its partial purification from colon 38 tumors grown subcutaneously in BDF_1 mice.

Purification. The purification scheme for TAD-phosphodiesterase from colon 38 tumor is presented in Table 3. Virtually all of the TAD-phosphodiesterase activity present in homogenates of the tumor was recovered in the high speed (105,000 *g*) supernatant fraction. Ammonium sulfate precipitation of the enzyme protein resulted in ~90% yield and 5-fold purification over the homogenate activity. Further purification of the ammonium sulfate enzyme on a DEAE-Sephadex column resulted in an overall purification of approximately 200-fold, and a recovery of 47% of the starting activity.

Table 2. Nucleotide pool size changes in sensitive and resistant tumors treated with tiazofurin (200 mg/kg)

Tumor	Time (hr)	GMP	GDP	GTP	UTP + CTP	IMP
				(% of Control)		
Colon 38	1	54	42	47	182	484
	2	58	43	53	218	630
	4	84	82	83	154	232
	6	95	100	97	113	108
	8	100	97	102	107	108
	24	95	90	104	99	112
P388	1	73	81	63	104	381
	2	51	74	47	103	841
	3	31	50	38	161	669
	4	12	45	33	173	644
	6	13	41	35	188	583
	8	33	60	47	241	677
	24	116	94	106	149	148

Male BDF_1 mice bearing either P388 or colon 38 tumors were injected intraperitoneally with tiazofurin (200 mg/kg). At indicated intervals tumors were removed, flash-frozen, and analyzed on HPLC for their nucleotide contents, as described in Materials and Methods. Each data point represents the mean of four animals; in no case did the individual values differ from the mean by more than 20%. Control values for the colon 38 tumor in nmoles/g (mean ± S.D.) were: GMP, 135 ± 45; GDP, 384 ± 48; GTP, 96 ± 24; UTP + CTP, 26 ± 4; and IMP, 22 ± 9. In the P388 tumor, these values were: GMP, 41 ± 13; GDP, 138 ± 27; GTP, 248 ± 52; UTP + CTP, 131 ± 35; and IMP, 16 ± 5.

Table 3. Purification of TAD-Phosphodiesterase

Fraction	Specific activity [μmoles · hr^{-1} · (mg protein)$^{-1}$]	Purification (fold)	Total activity [μmoles · hr^{-1} · (mg protein)$^{-1}$]	% Recovery
Homogenate	0.03	1.0	28.1	100
105,000 *g* Supernatant	0.06	2.0	27.4	98
Ammonium sulfate pellet	0.15	5.0	24.9	89
DEAE-Sephadex	6.32	211.0	13.3	47

TAD-Phosphodiesterase was purified from colon 38 tumors grown subcutaneously in BDF_1 mice. Enzyme activity was determined using [^{14}C]TAD as substrate; the product, [^{14}C]AMP, was measured after its separation on columns of Dowex 1-X8. Details of purification steps are presented in Materials and Methods.

The elution profile from the DEAE-Sephadex column is shown in Fig. 2. TAD-Phosphodiesterase eluted as a single symmetrical peak, at ~0.25 M NaCl. Alkaline phosphodiesterase activity, which was monitored with thymidine-5′-monophosphate-nitrophenyl ester as substrate, eluted just before, but separated from, the TAD-phosphodiesterase peak. Similar results were obtained in two separate purification runs. Resolution of these activities is supportive of the conclusion that TAD-phosphodiesterase can be excluded from the list of well-characterized phosphodiesterases which vigorously hydrolyze TMP-nitrophenyl ester [14–16]. Fractions containing the highest TAD-phosphodiesterase activity were pooled, dialyzed, and concentrated as detailed in Materials and Methods. This concentrated enzyme preparation was used for all subsequent studies. Not shown is the observation that TAD-phosphodiesterase activity was linear with protein concentration (1–20 μg/25 μl assay volume) and assay time (5–60 min).

Effect of pH on TAD/NAD degradation. Measurements of TAD-phosphodiesterase at three different pH values (7.6, 8.4 and 9.3) showed no significant change in the enzyme activity (Table 4). On the other hand, the TAD-hydrolyzing capacity of venom phosphodiesterase was affected markedly by hydrogen-ion concentration, resulting in greater than 4-fold increase in activity as the pH was raised from 7.6 to 9.3. Most noteworthy was the finding that NAD, the parent analog of TAD, proved to be extremely resistant to attack by TAD-phosphodiesterase (0–7% of TAD degradation) over the pH range tested. Venom phosphodiesterase, on the other hand, vigorously hydrolyzed NAD, but only at alkaline pH values.

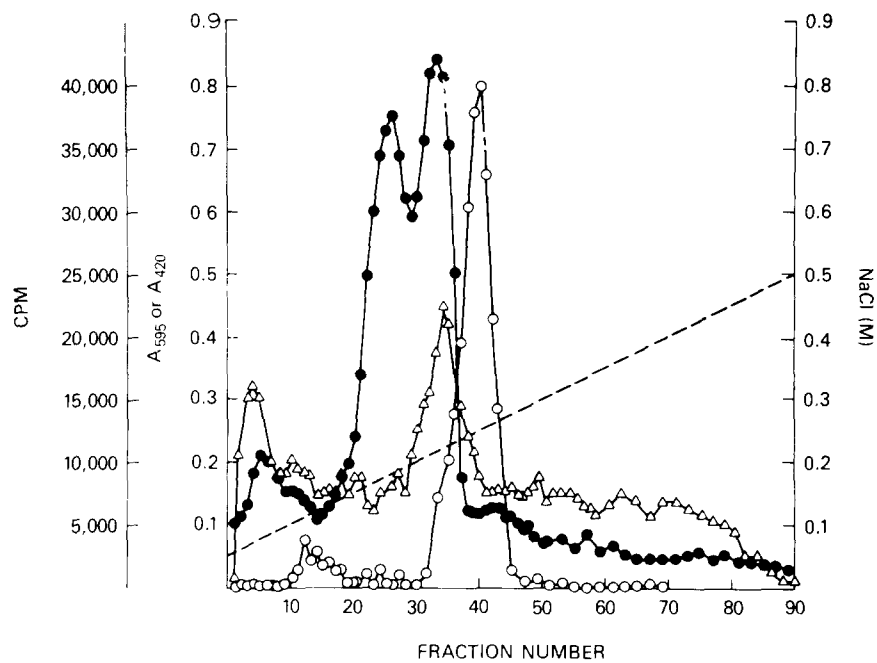

Fig. 2. TAD-Phosphodiesterase purification profile on DEAE Sephadex. TAD-Phosphodiesterase activity (○– – –○) was determined radiometrically using [^{14}C]TAD; alkaline phosphodiesterase activity (●– – –●) was monitored at 420 nm using TMP-nitrophenylester as substrate; and protein concentration (△– – –△) was measured using a Bio-Rad Kit, as described in Materials and Methods. A similar profile was observed in two separate purifications.

Table 4. Influence of pH on the degradation of TAD and NAD by TAD-Phosphodiesterase or snake venom phosphodiesterase

Enzyme	Substrate	Specific activity [μmoles · hr $^{-1}$ · (mg protein) $^{-1}$]		
		pH 7.6	pH 8.4	pH 9.3
TAD-Phosphodiesterase	NAD	<0.1	0.5	0.5
	TAD	7.0	7.6	6.9
Venom phosphodiesterase	NAD	<1.0	226.0	435.0
	TAD	194.0	795.0	894.0

TAD-Phosphodiesterase and venom phosphodiesterase activities were measured against either substrate (NAD or TAD) at the indicated pH values. Assays were conducted at a substrate concentration of 7.5 mM, and the hydrolytic products (NMN and AMP from NAD and AMP and TRMP from TAD) were isolated and measured on HPLC, as described in Materials and Methods. Each data point represents the mean of two separate analyses, showing less than 10% variation.

Kinetic analysis of the TAD-Phosphodiesterase activity—Effect on NAD concentration. The enzyme exhibited a rather low affinity for its substrate, TAD, yielding a K_m value of *ca.* 1.0 mM (Fig. 3). Although NAD was a poor substrate for TAD-phosphodiesterase (Table 4), it did inhibit TAD degradation in a competitive manner (Fig. 3). Over an NAD concentration range of 1.25 to 5.0 mM, the calculated average K_i value for NAD inhibition was ~4.5 mM.

Substrate preference of TAD-Phosphodiesterase toward pyrophosphate linkages.

(i) *Substrate properties.* In an attempt to identify the physiological substrate(s) for TAD-phosphodiesterase, a series of natural pyrophosphate-containing compounds was tested for substrate properties. As listed in Table 5, the rate of degradation of these compounds was compared with the pharmacological substrate, TAD. Of these structures, the preferred substrate was found to be ADP-ribose which was degraded at a rate slightly greater than that for TAD (111% of TAD degradation). Among the other natural TAD analogs, FAD, NADPH, NAAD and NHXD were all hydrolyzed at a rate approximately 25% that of TAD degradation. GDP-Mannose, UDP-glucuronic acid and CDP-choline were either not degraded or degraded at very minimal rates.

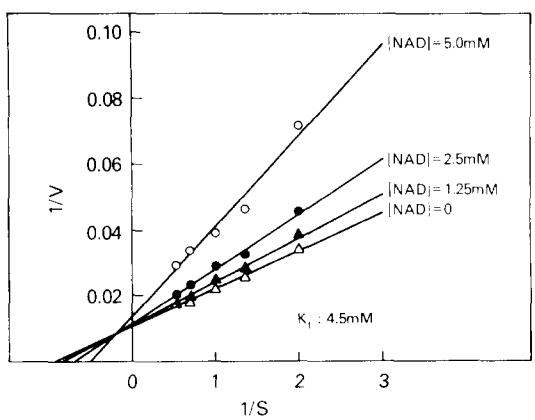

Fig. 3. TAD-Phosphodiesterase kinetics. TAD-Phosphodiesterase activity was determined at various concentrations of the substrate, TAD, and in the presence or absence of three concentrations of NAD, as indicated in this double-reciprocal plot. Enzyme activity was determined as described in Materials and Methods.

Among the parent analogs of TAD, the reduced form of NAD (NADH) was hydrolyzed at a rate similar to that for TAD (84% of TAD degradation), while the oxidized forms, NAD and NADP, were comparatively resistant to hydrolysis.

To investigate whether the presence of TAD could affect the decomposition of these natural substrates, their rate of degradation was measured *in vitro* in the presence and absence of TAD (Table 5). The degradation of ADP-ribose, which had been found to be an outstanding substrate for the enzyme, was inhibited by more than 40% in the presence of an equimolar concentration of TAD. The hydrolysis of other substrates with lesser susceptibilities to attack was inhibited to a variable degree by TAD: thus the breakdown of FAD was nearly fully repressed by TAD whereas the rate with NAAD and NADPH as substrates was only halved. Paradoxically, the degradation of NADH was not affected by the presence of TAD.

The activity of TAD-phosphodiesterase was also measured with substrates containing phosphodiester linkages and compared with the corresponding pyrophosphate substrate counterpart. To that end, synthetic purine dinucleotides containing a 5′ → 5′ pyrophosphate bond and dinucleotides connected via a 2′ → 5′ or 3′ → 5′ phosphodiester bridge were investigated as alternative substrates. As shown in Table 6, the most susceptible substrate, besides TAD, was A[5′]P-P[5′]A which yielded two moles of AMP per mole of substrate. Phosphate ester linkages such as those present in adenylyl (2′ → 5′)-adenosine (or guanosine) and adenylyl(3′ → 5′)guanosine appeared insensitive to the action of the enzyme, a result which strongly suggests that TAD phosphodiesterase is preferentially a 5′ → 5′ pyrophosphatase.

(ii) *Effect of phosphodiesters on TAD degradation.* The capability of the enzyme for hydrolyzing TAD was next tested in the presence of a representative group of pyrophosphate-containing compounds: TAD-phosphodiesterase activity was determined at

Table 5. Degradation of natural phosphodiesters by TAD-Phosphodiesterase in the presence or absence of TAD

Phosphodiester	Specific activity [μmoles · hr^{-1} · (mg protein)$^{-1}$]	% of TAD degradation	Degradation in the presence of TAD: Specific activity [μmoles · hr^{-1} · mg^{-1}]	Degradation in the presence of TAD: % of Control
TAD	7.5	100		
ADP-Ribose	8.3	111	4.70	57
FAD	2.0	27	ND*	0
GDP-Mannose	0.9	12	ND	0
UDP-Glucuronic acid	ND	0	ND	0
CDP-Choline	ND	0	ND	0
NAAD	2.1	28	1.10	53
NHXD	1.7	23	0.10	6
NADPH	1.7	23	0.50	30
NADH	6.3	84	6.50	103
NADP	0.2	3	0.06	31
NAD	0.2	3	0.16	82

The degradation rate of natural diesters was measured in the presence or absence of TAD. Enzyme assay was carried out using a 3 mM concentration of various diesters ± 3 mM TAD at a pH of 7.6. Hydrolytic products were quantitated using HPLC as described in the text. Each data point represents the mean of two separate analyses with less than 10% variation.

* Non-detectable (<0.01).

Table 6. Specificity of TAD-Phosphodiesterase

Substrate	Degradation [μmoles · hr^{-1} · (mg protein)$^{-1}$]	% Degradation compared to TAD
A[5′]P-P[5′]T (TAD)	7.7 ± 0.05*	100.0
A[5′]P-P[5′]A	2.34 ± 0.02	30.0
A[2′]P[5′]A	0.20 ± 0.01	2.6
A[2′]P[5′]G	0.50 ± 0.02	6.5
A[3′]P[5′]G	0.15 ± 0.08	2.0

Dinucleotides containing 5′ → 5′ diphosphate linkage, 2′ → 5′ phosphate linkage or 3′ → 5′ phosphate linkage were subjected to TAD-phosphodiesteratic hydrolysis, at a substrate concentration of 7.5 mM. Products formed were quantitated by HPLC, as described in Materials and Method. Abbreviations: A, adenosine; G, guanosine; T, thiazole-4-carboxamide; and P, phosphate.

* Mean ± standard deviation of triplicate measurements.

Table 7. Inhibition of the degradation of TAD by representative phosphodiesters

Inhibitor	Degradation of TAD: Specific activity [μmoles · hr^{-1} · (mg protein)$^{-1}$]	Degradation of TAD: % of Control
None	7.5	100
ADP-Ribose	3.0	40
FAD	6.5	87
GDP-Mannose	7.5	100
UDP-Glucuronic acid	7.5	100
CDP-Choline	7.5	100
NAAD	6.5	87
NHXD	6.5	87
NADPH	7.5	100
NADH	5.0	67
NADP	7.0	93
NAD	5.0	67

The degradation of TAD by TAD-phosphodiesterase was measured in the presence or absence of the above-listed natural diesters at a concentration of 3 mM each. Hydrolytic products were quantitated on HPLC as described in Materials and Methods. Each data point represents the mean of two separate analyses, with less than 10% variation.

a TAD concentration of 3 mM, in the presence or absence of equimolar concentrations of the listed compounds (Table 7). Most important in this table is the inhibition produced by ADP-ribose, which inhibited TAD degradation by up to 60%. Both NAD and NADH at the concentration used inhibited TAD degradation by ~33%, whereas NADP and NADPH had no effect on this hydrolysis. Other pyrophosphates were also found to have little or no influence (~13% inhibition) on TAD degradation.

DISCUSSION

The importance of a cell's TAD-synthetic capacity (probably catalyzed by NAD pyrophosphorylase) to the therapeutic activity of tiazofurin has been clear since our earlier observation that mutant P388 lymphoblasts resistant to the drug were characterized by a nearly total deletion of this enzyme. More recently it has become obvious that the capacity to degrade TAD was also a decisive determinant of resistance to tiazofurin, both in a panel of murine tumors grown *in vivo* [8] and in six human lung tumor cells growing continuously in culture [10]. Without exception, resistance was accompanied in these cases by a substantial enhancement in the specific activity of an as yet unidentified enzyme or enzymes which decomposed TAD via cleavage of its phosphodiester linkage. The result of this degradation can be perceived in Fig. 1 and Table 2: depletion of TAD relieves the inhibition of IMPD engendered by the dinucleotide, and ultimately permits restitution of the GXP pools. The present series of experiments were undertaken in an attempt to characterize this degradative activity more extensively.

Toward this end, the enzyme has been partially purified from nodules of the colon 38 carcinoma, a line solidly resistant to tiazofurin. While not homogeneous, the enzyme so purified has been a valuable tool for establishing the essential features of TAD breakdown. The enzyme is a pyrophosphatase with little activity toward either thymidine-5′-monophosphate nitrophenyl ester (Fig. 2) or the prototypical cellular dinucleotide, NAD, at least at physiological pH (Table 4). A corollary of this fact is our finding that NAD was not a potent inhibitor of TAD degradation *in vitro* (K_i 4.5 mM), and thus it is unlikely that it will regulate the activity of the enzyme *in vivo*, especially when the concentration of all four pyridine nucleotides is much lower than this constant [17]. By contrast, ADP-ribose is a good substrate for the enzyme at physiologic pH, and the strongest naturally occurring inhibitor detected in our studies *in vitro*. This potency notwithstanding, it is doubtful that ADP-ribose can attain the requisite concentrations *in vivo* to repress the degradation of TAD in a pharmacologically important way. Nevertheless *in vivo* it may be the natural or preferred substrate for the enzyme we have designated TAD-phosphodiesterase.

From the results presented in this report, and from our previous work [8, 10], we have learned that resistance to tiazofurin correlates well with TAD-phosphodiesterase activity. To alter the resistant nature of tumors, it will be necessary either to synthesize analogs of TAD which are more resistant to such cleavage, or to prolong the intracellular half-life of TAD by inhibiting this degradative enzyme. Future reports from our laboratories will examine such possibilities.

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