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Effects of 2-Amino-1,3,4-thiadiazole on Ribonucleotide Pools of Leukemia L1210 Cells[1]

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SUMMARY

The effects of 2-amino-1,3,4-thiadiazole [aminothiadiazole (NSC 4728)] on purine and pyrimidine ribonucleotide pools of L1210 ascites cells *in vivo* are presented and discussed as they relate to the site of action. Within 1 hr after administration of the drug, the levels of guanosine triphosphate, guanosine diphosphate, adenosine triphosphate, and adenosine diphosphate were reduced, whereas those of inosine monophosphate (IMP) and uridine triphosphate were increased. The most pronounced effects were the lowering of guanine ribonucleotide pools and the elevation of IMP. Aminothiadiazole produced a marked inhibition (~95%) of the incorporation of [8-^{14}C]inosine into guanine nucleotides, whereas only a slight inhibition (~20%) of incorporation into adenine nucleotides was observed. These results suggest that the thiadiazole (or a metabolite thereof) inhibits the conversion of IMP to guanosine monophosphate; this conclusion is reinforced by the observation that mycophenolic acid, a known inhibitor of this conversion, produced effects on ribonucleotide pools similar to those produced by aminothiadiazole. Aminothiadiazole did not inhibit IMP dehydrogenase isolated from L1210 cells. The effects of the thiadiazole on nucleotide pools were prevented by simultaneous administration of nicotinamide. Since nicotinamide is known to prevent or reverse the antileukemic activity of aminothiadiazole, it is probable that the inhibition of synthesis of guanosine monophosphate is related to the antileukemic action of this agent.

INTRODUCTION

2-Amino-1,3,4-thiadiazole, NSC 4728 (referred to herein as aminothiadiazole) and certain structurally related compounds have been known for 20 years to have antitumor activity (16). Compounds of this class are uricogenic agents in man, and this effect appears to result from a stimulation of purine synthesis *de novo* (9–11). Both the antitumor and the uricogenic activities can be prevented or reversed by nicotinamide (5, 9–11, 15, 18). One derivative, 2-ethylamino-1,3,4-thiadiazole, has been shown to exchange with the nicotinamide moiety of NAD in the presence of porcine NADase (5). It is possible, therefore, that an analog of NAD is responsible for some of the biological effects of aminothiadiazole. A recent report indicates that these compounds may elevate 5-phosphoribosyl 1-pyrophosphate levels in mouse liver, an effect which might be related to stimulation of purine biosynthesis (12). The results reported herein point to a blockade of the synthesis of guanine nucleotides, presumably at the enzymatic step, IMP to XMP, or, possibly, XMP to GMP, by aminothiadiazole metabolite(s). After work reported herein had been completed, Tsukamoto *et al.* (21) arrived at the same conclusion concerning the site of action of the related compound, 2,2′-(methylenediimino)bis-1,3,4-thiadiazole, in the mouse cell line BALB/3T3 using cytotoxicity reversal and radioactive precursor uptake techniques.

MATERIALS AND METHODS

L1210/0 cells were maintained by weekly transfer of cells harvested from the i.p. culture in C57BL × DBA/2 F_1 (hereafter called $BD2F_1$) mice as has been described previously for this laboratory (19). Drugs were administered i.p. to mice that had received implants of 10^5 cells 6 days earlier. Cells were harvested by washing from the peritoneal cavity with Earle's solution. Red blood cells were removed by lysis with isotonic NH_4Cl solution (3). Extraction of the cells with 0.5 N perchloric acid was performed essentially as reported by Scholar *et al.* (17).

Levels of ribonucleotide pools were determined in the acid-soluble, neutralized extracts by means of the high-pressure liquid chromatograph described previously (13). A Partisil-10 SAX (H. Reeve Angel & Co., Inc., Clifton, N. J.) anion-exchange column (4.6 mm i.d. × 25 cm) at ambient temperature was used. A linear gradient (40 min) from 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, was used. The flow rate was 2 ml/min, and detection of eluting materials was accomplished by UV absorbance at 254 or 280 nm. Integration of peak areas was performed by a Hewlett-Packard Model 3380A digital electronic integrator, and quantitation was achieved using the peak area per nmole relationship obtained with commercially supplied ribonucleoside monophosphates. Verification of IMP in extracts of L1210 cells treated with aminothiadiazole was accomplished by separation and quantitation with 2 additional high-pressure liquid chromatographic systems, namely: *(a)* continuous elution (1 ml/min; 5 mM KH_2PO_4, pH 3.5) of a Bondapak AX (2.3 mm x 61 cm) column at ambient temperature coupled to a pellicular anion-exchange column (1 mm i.d. x 305 cm) at 80° (14); and *(b)* continuous elution of a reverse-phase column (μBondapak C_{18}, Waters Associates, Milford, Mass.; 4 mm x 30 cm) with 100 mM $NH_4H_2PO_4$, pH 3.5, at 2 ml/min.

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Table 1

Effects of aminothiadiazole on ribonucleotide pools of L1210 in vivo

L1210/0 cells (10^5) were implanted by injection into the peritoneal cavity of $BD2F_1$ mice. Six days later the animals, in groups of 5, were treated i.p. with the agents shown. Four hr after treatment, the ascites tumor cells were removed, washed with cold Earle's solution, freed of red blood cells with isotonic NH_4Cl, and extracted with cold 0.5 N perchloric acid. The neutralized extracts were analyzed by high-pressure liquid chromatography.

	Levels (nmoles/10^9 cells) after treatment with						
		Aminothiadiazole					
	Control (10)[a]	3 mg/kg (1)	10 mg/kg (1)	100 mg/kg (7)	Nicotinamide, 100 mg/kg (3)	Aminothiadiazole + nicotinamide, 100 mg/kg each (3)	Mycophenolic acid, 100 mg/kg (3)
ADP	151 ± 20	96	71	64 ± 6	124 ± 2	112 ± 16	118 ± 3
ATP	573 ± 29	536	360	328 ± 36	472 ± 18	516 ± 38	407 ± 56
GDP	40 ± 5	21	8	11 ± 2	29 ± 2	19 ± 3	16 ± 1
GTP	132 ± 8	96	26	25 ± 2	105 ± 7	53 ± 6	24 ± 1
CTP	24 ± 3	17	19	26 ± 5	19 ± 2	22 ± 2	32 ± 2
UTP	158 ± 12	135	227	238 ± 33	125 ± 4	206 ± 14	262 ± 28
IMP	10 ± 1	32	145	127 ± 16	10 ± 1	48 ± 22	97 ± 3
NAD	90 ± 15	64	79	90 ± 8	87 ± 15	83 ± 8	96 ± 16

[a] Numbers in parentheses, number of separate experiments.

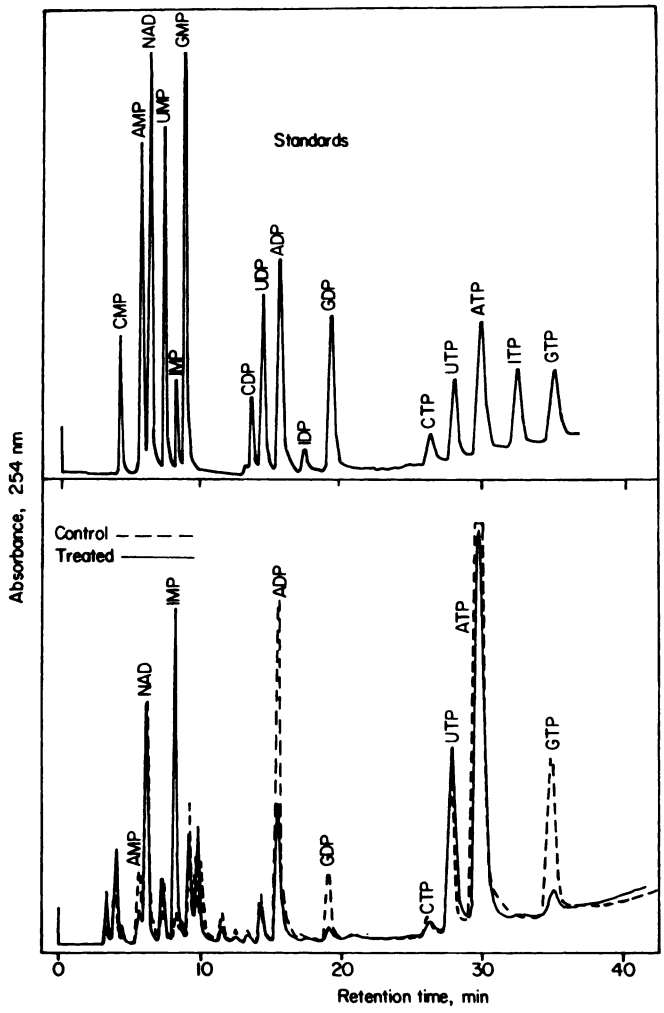

Chart 1. Purine and pyrimidine ribonucleotide pools of L1210 cells treated with aminothiadiazole. The neutralized, acid-soluble extracts of approximately 10^7 tumor cells obtained from control mice or from mice treated 4 hr previously with aminothiadiazole (100 mg/kg) were subjected to analysis for nucleotide content by means of the high-pressure liquid chromatograph described in "Materials and Methods." *Upper*, separation obtained with approximately 1 nmole of each of the authentic ribonucleotides.

Using the 3 separation modes described above (Partisil-10 SAX, pellicular anion, and reverse phase), identical results for IMP levels were obtained for 6 of the samples given in Table 1. Using the 3 methods, the range of values for IMP was less than 10% for each sample. Attempts to verify IMP by enzymatic peak shift (4) using IMP dehydrogenase were not entirely successful due to inhibition by the product XMP (1, 8). The levels of NAD in 26 samples shown in Table 1 were verified by enzymatic analysis using alcohol dehydrogenase (2).

IMP dehydrogenase was prepared essentially as described by Franklin and Cook (6) using L1210/0 ascites cells harvested from about 50 mice (~10 g of cells). A 2% solution of streptomycin instead of protamine sulfate was used to precipitate RNA, and the initial 60% ammonium sulfate precipitation was not used. Details of the assay are given in Table 3.

Purine and pyrimidine ribonucleotides were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Aminothiadiazole was supplied by the Drug Research and Development Division of the National Cancer Institute. [8-^{14}C]Inosine (35 μCi/μmole) from Schwarz/Mann, Orangeburg, N. Y., and [8-^{14}C]guanine (57 μCi/μmole) from New England Nuclear, Boston, Mass., were used for radioactive precursor experiments as reported previously (13).

RESULTS AND DISCUSSION

Treatment of L1210-bearing mice with aminothiadiazole reduced adenine and guanine ribonucleotide pools and increased pools of UTP and IMP in the tumor cells (Chart 1). The level of NAD was unaltered, as were the levels of the unidentified components eluting at 9 to 10 min. The most marked changes observed were the decrease in guanine nucleotide levels to about 20% of control and the increase in IMP. The effects on UTP, ADP, and ATP levels may be secondary to the reduction of the pools of GTP, since GTP is required for the synthesis of AMP from IMP and for the synthesis of CTP from UTP.

The effects of aminothiadiazole on ribonucleotide pools occur within 1 hr after a dose of 100 mg/kg, and they persist for at least 4 hr (Chart 2). Recovery from effects on IMP, UTP, and GTP levels is apparent 24 hr after drug administration.

The quantitative effects of aminothiadiazole on ribonucleotide pools are summarized in Table 1. A drug dose of 3 to 10 mg/kg was required to produce the above-mentioned alterations in nucleotide levels. Nicotinamide given simultaneously with aminothiadiazole prevented to a large degree the effects on IMP, ADP, and ATP, and to a lesser degree prevented the effects on GDP, GTP, and UTP. The alterations in nucleotide pools produced by aminothiadiazole are consistent with a blockade in the conversion of IMP to GMP. Mycophenolic acid, a known inhibitor of this conversion (20), produced similar effects on ribonucleotide pools (Table 1). To obtain more direct information concerning the effect of aminothiadiazole on GMP synthesis, experiments were performed with ^{14}C-labeled inosine and guanine (Table 2). Treatment with aminothiadiazole reduced the incorporation of radioactive inosine into guanine ribonucleotide pools by about 95%, and the incorporation into adenosine nucleotides from [8-^{14}C]inosine was reduced by only 18%. Incorporation of radioactive guanine into guanine nucleotides was also reduced by aminothiadiazole; however, accumulation of radioactivity in the monophosphate area of the chromatogram did not occur. This finding suggests that the effect of aminothiadiazole on guanine utilization may occur at an earlier step, *i.e.*, hypoxanthine-guanine phosphoribosyltransferase. It would appear likely that the inhibition of [8-^{14}C]guanine utilization occurs due to the high intracellular levels of IMP (~100 μM, Table 1) that accumulate after treatment with this drug. IMP is known to inhibit the hypoxanthine-guanine phosphoribosyltransferase required to form GMP from guanine (7). Mycophenolic acid mimicked the effects of aminothiadiazole on precursor labeling (data not shown); however, incorporation of radioactive inosine into adenosine ribonucleotides was increased by mycophenolic acid as was observed in studies in H.Ep. No. 2 cells using [8-^{14}C]hypoxanthine as the precursor (13).

The inhibition of the IMP to GMP conversion could result from a blockade of IMP dehydrogenase, a blockade of GMP synthetase, or from some other blockade resulting in an inhibition of GMP synthesis as a secondary effect. IMP dehydrogenase would appear to be the most likely site of action because of the nicotinamide-thiadiazole relationship and the fact that NAD is a cofactor for this reaction. Ami-

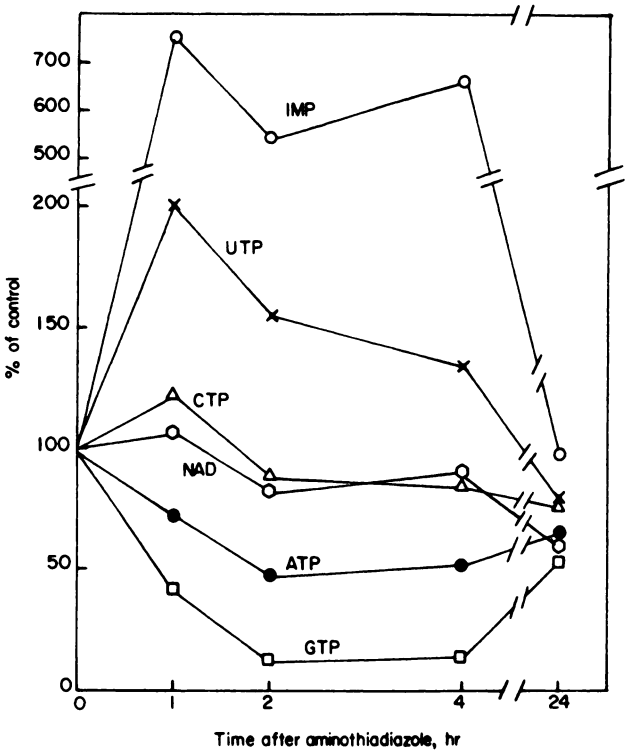

Chart 2. Time course of the effects of aminothiadiazole on ribonucleotide pools of L1210 cells. Mice bearing the ascites tumor were treated i.p. with 100 mg of aminothiadiazole per kg. At 1, 2, 4, and 24 hr later, tumor cells were harvested and extracted with perchloric acid. Ribonucleotide pools were determined in the neutralized extracts as shown in Chart 1. The values given are average results obtained in 2 separate experiments. Alterations in IMP pool size underestimate the absolute changes, since an arbitrary control level of 30 nmoles/10^9 cells was used. The actual control value is lower than 30 nmoles/10^9 cells, but is difficult to assess precisely by the method used in cells not treated with aminothiadiazole (see Table 1).

Table 2

Effects of aminothiadiazole on the incorporation of radioactive precursors by L1210 cells

Groups of 5 mice bearing L1210 implants were pretreated for 15 min with aminothiadiazole, 100 mg/kg i.p. One hr after administration of the radioactive precursors (1 μCi/mouse, i.p.), cells were harvested, extracted, and the radioactivity associated with ribonucleotides in 2 × 10^7 cells was determined by collecting fractions of the eluant from the chromatograph, as shown in Chart 1.

	Observed cpm		
	ADP + ATP	GDP + GTP	Monophosphates
[8-^{14}C]Inosine			
Control	4939	760	845
Treated	4065	37	1191
[8-^{14}C]Guanine			
Control	461	2162	733
Treated	454	762	166

Table 3

Lack of effect of aminothiadiazole on IMP dehydrogenase isolated from L1210 ascites cells

The conversion of NAD to NADH was followed at 340 nm in 1-ml cuvets containing the substrates or inhibitors in 100 mM Tris-Cl buffer at pH 7.4 and 37°. The concentration of K^+ was 100 mM, and the reaction was initiated by the addition of IMP. Inhibitors were added during the period in which NADH formation was linear with respect to time. The amount of enzyme preparation per reaction was 2 mg protein (from 400 μl of L1210 cells).

	Enzyme activity (nmole/min/mg protein)
NAD, 0.3 mM; IMP, 2 mM:	
Control	0.77
+ aminothiadiazole, 2 mM	0.70
+ mycophenolic acid, 0.1 mM	0.11
NAD 0.3 mM; IMP 0.2 mM:	
Control	0.71
+ aminothiadiazole, 2 mM	0.77
+ mycophenolic acid, 0.1 mM	0.17
NAD, 0.03 mM; IMP, 0.2 mM:	
Control	0.35
+ aminothiadiazole, 2 mM	0.36

nothiadiazole did not inhibit IMP dehydrogenase (Table 3), but this fact does not eliminate the dehydrogenase as a site of action, because a metabolite of aminothiadiazole may be the active form of this agent. Elucidation of the mechanism of the blockade of GMP synthesis must await identification of the metabolites (if any) of aminothiadiazole and their study as inhibitors of IMP dehydrogenase and GMP synthetase. Such studies are under way.

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