Mechanism of Action of 2-Amino-1,3,4-thiadiazole (NSC 4728) 1

J. Arly Nelson, 2 Lucy M. Rose, and L. Lee Bennett, Jr. 3
Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205

SUMMARY

The synthesis and isolation of two derivatives of 2-amino-1,3,4-thiadiazole (aminothiadiazole) are described. The derivatives are a nicotinamide adenine dinucleotide (NAD) analog prepared by an exchange reaction with NAD in the presence of nicotinamide adenine dinucleotide glycohydrolase and a presumed aminothiadiazole mononucleotide prepared by treatment of the NAD analog with nucleotide pyrophosphatase. Both derivatives are potent inhibitors of inosine 5'-phosphate (IMP) dehydrogenase obtained from leukemia L1210 cells. The NAD analog is a pseudoirreversible inhibitor of the enzyme, noncompetitive with either IMP or NAD. The aminothiadiazole mononucleotide has a K1 of about 0.1 μM, is competitive with IMP, and is uncompetitive with NAD; the inhibition appears to be reversible by Ackermann-Potter analysis. A metabolite of [5-14C]aminothiadiazole is formed in L1210 cells in vivo to a level of 0.3 nmole/106 cells. Retention volume of the metabolite on a high-pressure liquid chromatography system is the same as that of the aminothiadiazole mononucleotide prepared as described above. These results suggest that IMP dehydrogenase is the site of action for aminothiadiazole metabolites as indicated by earlier observations.

There is no evidence that the NAD analog is formed in vivo. Nicotinamide prevented formation of the mononucleotide in vivo. Therefore, since formation and cleavage of the NAD analog apparently are not the route to the thia diazole nucleotide, some other pathway for the metabolism of nicotinamide may be involved such as the action of a phosphoribosyltransferase or the sequential action of a nucleoside phosphorylase and a nucleoside kinase.

INTRODUCTION

Aminothiadiazole 4 (Chart 1) and certain of its derivatives are of interest because of their antitumor and uricogenic activities, both of which are prevented by nicotinamide (7, 11). 2-Ethylaminothiadiazole has been shown to exchange with the nicotinamide moiety of NAD in the presence of a mammalian NADase (7). Although these observations would indicate the NAD analogs as possible active metabolites of the aminothiadiazoles, no NAD analog has been isolated from cells exposed to these compounds nor has a NAD analog of an aminothiadiazole been evaluated as an inhibitor of any of the NAD-requiring enzymes. Recent studies from this (13) and another laboratory (18) have provided evidence, by the use of different experimental techniques, that metabolites of aminothiadiazole and of the closely related compound, 2,2’-(methyleneimino)bis-1,3,4-thiadiazole (NSC 143019), produce inhibition of tumor cells by blocking the formation of guanine nucleotides from IMP. Of the 2 enzymes involved in the IMP to GMP conversion, IMP dehydrogenase (EC 1.2.1.14) is the more likely site of action since it requires NAD. We report here the enzymatic synthesis of 2 aminothiadiazole derivatives that inhibit IMP dehydrogenase. Preliminary data suggest that one of the derivatives may be formed following treatment of L1210 cells with aminothiadiazole in vivo. A preliminary report of this work has been presented (12).

MATERIALS AND METHODS

Materials. NADase (EC 3.2.2.5) from hog brain, nucleotide pyrophosphatase (EC 3.6.1.9) from Crotalus adamanteus venom, and 5'-adenylic acid deaminase (EC 3.5.4.17) were obtained from Sigma Chemical Co., St. Louis, Mo. [5-14C]Aminothiadiazole (6.5 μCi/μmole) and nonradioactive aminothiadiazole were obtained from the Drug Research and Development Division of the National Cancer Institute. Other chemicals and nucleotides were obtained from Sigma or from P-L Biochemicals, Milwaukee, Wis.

Preparation of the Aminothiadiazole Analog of NAD. [5-14C]Aminothiadiazole (200 μmole; 5 to 36 nCi/μmole) was incubated at 37° for 3 to 4 hr in the presence of 60 μmole of NAD, 100 mg (1.5 units) of hog brain NADase, and 10 mm Tris-Cl, pH 7.4, in a total volume of 5 ml. The reaction was terminated by addition of 0.1 volume 50% trichloroacetic acid. After centrifugation to remove the precipitated protein, 5 volumes of ice-cold acetone were added and the samples were stored overnight at −20°. The precipitate was washed 4 times with ice-cold acetone and then dissolved in 1 ml of 10 mm Tris-Cl, pH 7.4. This procedure is essentially that used by Cioti et al. (7) to prepare the NAD analog of 2-ethylamino-1,3,4-thiadiazole. The entire sample was then injected onto a Partisil-10 SAX column (4.6 mm inside diameter x 25 cm; Whatman Inc., Clifton, N. J.), and the radioactive NAD analog was purified using the ammonium acetate system described below. The extent of aminothiadiazole exchanged was less than 1% of the NAD present in the above reaction; the procedure was scaled up 5-fold in order
to obtain larger amounts of the NAD analog.

**Preparation of Thiadiazole Mononucleotide.** Approximately 0.4 μmole of the NAD analog prepared as described above was treated with 1 unit of nucleotide pyrophosphatase in the presence of 5 mM MgCl₂ and 10 mM Tris-Cl at pH 7.4 in a total volume of 1 ml for 90 min at 37°C. As determined by UV absorption at 254 nm of portions subjected to high-pressure liquid chromatography using the ammonium phosphate system, conversion was essentially complete under these conditions. After incubation, the entire sample was placed on the Partisil-10 SAX column, and thiadiazole mononucleotide was obtained by collecting fractions of the eluant using the ammonium acetate system described below.

**High-Pressure Liquid Chromatography.** A Waters Associates ALC/202 liquid chromatograph equipped with 2 Model 6000 pumps and a Model 660 gradient programmer was used as described previously (13). Two gradients were employed using the Partisil-10 SAX column. An ammonium phosphate gradient system was used for analytical purposes (13). The gradient was from 5 mM NH₄H₂PO₄ at pH 2.8 to 750 mM NH₄H₂PO₄ at pH 3.7 in 40 min at a flow rate of 2 ml/min. An ammonium acetate gradient system was used to prepare the aminothiadiazole derivatives, since this salt could be removed without degradation of the analog nucleotides by evaporation with an Evapo-Mix (Buchler Instruments, Fort Lee, N. J.) at 35–40°C under reduced pressure. The gradient was from 250 mM ammonium acetate at pH 4.7 to 1 mM ammonium acetate at pH 4.7 formed in 10 min at a flow rate of 2 ml/min. Fractions of the eluant were collected at 1-min intervals, and portions were taken for radioactivity determinations by liquid scintillation spectrometry to locate the [5-¹⁴C]aminothiadiazole derivatives. The ammonium acetate system, unlike the ammonium phosphate system, provided facile separation of the NAD analog from the major reaction product, adenosine diphosphoribose, and also afforded good separation of the thiadiazole mononucleotide from the NAD analog (Table 1). The aminothiadiazole derivatives prepared in this manner were determined to be >90% pure by rechromatography using the phosphate eluting system.

**Preparation of IMP Dehydrogenase from L1210 Cells.** L1210 cells were harvested from the peritoneal cavities of 50 mice on Day 6 following implantation of 10⁶ cells. Generally, about 10 g of cells were obtained after washing with Earle’s solution and lysis of RBC using isotonic NH₄Cl (4). The cells were disrupted at a concentration of 30% in 100 mM Tris-Cl at pH 7.4 by sonic oscillation. The disrupted cells were subjected to centrifugation at 10,000 x g for 20 min followed by further centrifugation at 105,000 x g for 60 min. The 105,000 x g supernatant was strained through 2 layers of cheesecloth, and streptomycin sulfate was added to a concentration of 2%. The precipitate was removed by centrifugation, and an ammonium sulfate precipitate formed between 25 and 45% saturation was prepared for use as a source of IMP dehydrogenase. The precipitate obtained at 45% saturation of ammonium sulfate was dissolved in 10 mM Tris-Cl (1 ml for each 2 g of original packed cell volume) and was dialyzed overnight against 1 liter of 10 mM Tris-Cl, pH 7.4. The small amount of precipitate formed during dialysis was removed by centrifugation, and the preparation was stored at 4°C and used within 3 days. The preparation is a modification of those described previously for other mammalian tissues (3, 10). Analysis of the enzyme was performed at 37°C in 1-ml cuvets by following the formation of NADH at 340 nm using a Beckman Model DU spectrophotometer with Gilford attachments. The results shown (Charts 3 and 4) are representative of 2 or more experiments in which results agreed within 10%.

### RESULTS

The NAD analog, prepared from [5-¹⁴C]aminothiadiazole as described above, was characterized by shifts of peaks (determined by high-pressure liquid chromatography) after treatment with various enzymes. NAD was subjected to the same treatments and served as a model compound to assure that the “peak shift” (5) conditions were appropriate. These results are shown in Chart 2. Since the NAD analog was prepared from aminothiadiazole of known specific activity, the amount of aminothiadazole present in the NAD analog was determined by radioassays (1.4 nmoles). Treatment of the NAD analog with nucleotide pyrophosphatase resulted in essentially complete disappearance of the peak for the NAD analog and the appearance of 2 new peaks, one with the retention time of AMP and the other (Chart 2, TMN) presumed to be a nucleotide of aminothiadiazole. After treatment with adenylate deaminase, the AMP disappeared and a peak with the retention time of IMP appeared; the thiadiazole mononucleotide peak was unaltered by the deaminase. Under the same series of treatments, NAD was
After Nucleotide Pyrophosphatase

<table>
<thead>
<tr>
<th>NAD Analog</th>
<th>NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 nmoles</td>
<td>1.3 nmoles</td>
</tr>
</tbody>
</table>

After Nucleotide Pyrophosphatase Plus Adenylyl Deaminase

<table>
<thead>
<tr>
<th>IMP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 nmoles</td>
<td>15 nmoles</td>
</tr>
</tbody>
</table>

Chart 2. Verification of the aminothiadiazole analog of NAD by enzymatic peak shift and illustration of the method used to prepare thiadiazole mononucleotide (TMN). The NAD analog was prepared by reaction with NAD in the presence of NADase and was purified by liquid chromatography. In the separations shown above, the ammonium phosphate elution system was used to follow the conversion of NAD or of the NAD analog to AMP and nicotinamide mononucleotide (NMN) or thiadiazole mononucleotide by nucleotide pyrophosphatase. Subsequent treatment with adenylyl deaminase quantitatively converted the AMP to IMP. The small peak at 9 min is probably residual adenosine diphosphoribose. No compounds were eluted after 12 min.

Chart 3. Kinetics of the inhibition of IMP dehydrogenase by derivatives of aminothiadiazole. Enzyme from L1210 ascites tumor was assayed in the presence or absence of inhibitors at various concentrations of IMP. The concentrations of other factors were: 70 μM NAD; 100 mM KCl; 50 mM EDTA; 100 mM Tris-Cl, pH 7.4. The reaction was initiated by the addition of approximately 0.5 mg of enzyme protein. •, control; ○, NAD analog, 9 μM; Δ, thiadiazole mononucleotide, 0.1 μM.

Chart 4. Inhibition of IMP dehydrogenase by derivatives of aminothiadiazole at various concentrations of NAD. The enzyme reaction was followed spectrophotometrically in the presence or absence of the inhibitors. Concentrations of other factors in the reaction mixture were: 200 μM IMP; 100 mM KCl; 50 mM EDTA; 100 mM Tris-Cl, pH 7.4. The reaction was initiated by the addition of approximately 0.5 mg of enzyme protein. •, control; ○, NAD analog, 9 μM; Δ, thiadiazole mononucleotide, 0.2 μM.

Neither the NAD analog nor thiadiazole mononucleotide behaves like a competitive inhibitor with respect to the substrate, NAD (Chart 4). Inhibition by the NAD analog is noncompetitive and that by the thiadiazole nucleotide apparently is uncompetitive. The concentration of IMP (200 μM) used in these experiments was chosen to be well in...
excess of the $K_m$ values (20 to 30 $\mu$M) reported for IMP dehydrogenase preparations from mammalian sources (3, 10). By Ackermann-Potter analysis (1), the NAD analog behaves like a pseudoirreversible inhibitor, whereas inhibition by thiadiazole mononucleotide appears to be reversible (Chart 5).

To determine whether either of these enzymatically prepared aminothiadiazole derivatives was formed in vivo, [5-$^{14}$C]aminothiadiazole was administered to mice bearing L1210 cells, and extracts of the cells were analyzed by high-pressure liquid chromatography with the ammonium acetate system (Chart 6). Two radioactive compounds were present in the extracts. One with a retention time of about 3 min presumably is unchanged aminothiadiazole. The 2nd peak of radioactivity appeared at 19 to 20 min, corresponding to the retention time of thiadiazole mononucleotide (Table 1). There was no evidence for the presence of the NAD analog (retention time, ~15 min) in the cell extracts. Nicotinamide, administered to the animals at the same time as [5-$^{14}$C]aminothiadiazole, had no effect on the 1st peak but prevented completely the formation of the compound eluting at ~19 min. In similar experiments not shown, nicotinic acid, hypoxanthine, adenine, or guanine were either ineffective or were much less effective than nicotinamide in preventing formation of the metabolite. The level of the metabolite eluting at 19 to 20 min in L1210 cells from mice treated with aminothiadiazole alone at 5 mg/kg is about 0.3 nmole/10$^6$ cells.

**DISCUSSION**

Earlier results (13, 18) have indicated inhibition of IMP dehydrogenase as the likely mechanism for inhibition of cellular proliferation by aminothiadiazole and related compounds. The results of the present study are consistent with this mechanism since 2 derivatives of aminothiadiazole, formed in vitro by enzymes occurring in mammalian cells, are shown to be potent inhibitors of this enzyme. The 2 derivatives are an aminothiadiazole analog of NAD and a compound derived from it by the action of nucleotide pyrophosphatase and hence presumed to be the 5'-phosphate of a ribonucleoside of aminothiadiazole. Since both of these compounds have $K_i$ values for IMP dehydrogenase in the micromolar range, the production of either in the intact cell could account for the observed effects on the synthesis of GMP. However, the aminothiadiazole nucleotide appears at present more likely to be the inhibitory metabolite because L1210 cells exposed to [$^{14}$C]aminothiadiazole in vivo contained no detectable amount of the analog of NAD but did contain a $^{14}$C-labeled metabolite with the same retention time on a Partisil-10 SAX column as that of the presumed aminothiadiazole nucleotide prepared by the action of nucleotide pyrophosphatase on the NAD analog. The aminothiadiazole nucleotide is a much more potent inhibitor of IMP dehydrogenase than is the NAD analog (Charts 3 and 4); the ratio, $K_i$ (IMP):$K_i$ (thiadiazole mononucleotide), is 360. Furthermore, after in vivo administration of a near-therapeutic amount of aminothiadiazole (5 mg/kg), a metabolite which may be aminothiadiazole nucleotide was found in L1210 cells at a concentration of about 0.3 $\mu$M. If this metabolite is in fact identical with the aminothiadiazole nucleotide prepared enzymatically, then this concentration should produce inhibition of the enzyme in vivo.

These initial findings on the nature of the inhibition of IMP dehydrogenase may be subject to some revision in the future, especially as concerns the NAD analog, since it behaves like a pseudoirreversible inhibitor (Chart 5). Calcu-
lation of the $K_i$ for inhibition of this nature requires a type of analysis beyond that reported herein (6). The apparent uncompetitive nature of the inhibition produced by the thia-
diazole nucleotide with respect to NAD (Chart 4) is consistent with its interaction with the enzyme at the IMP binding site (3), and it is possible that the inhibition would have been clearly noncompetitive had a higher concentration of thia-
diazole nucleotide been used (9).

The NAD analog has been more thoroughly characterized than has the aminothiadiazole mononucleotide derived therefrom. Thus, the NAD analog has been shown to con-
tain a thia diazole:ribose:phosphate ratio of 1.0:2.0:1.7 and to be cleaved by nucleotide pyrophosphatase to (a) AMP and (b) a compound that contains aminothiadiazole and that has a retention time on a Partisil column in the range of those of other nucleoside monophosphates. A question as yet unanswered for both compounds is the point of attachment of the ribosyl group to the thia diazole. Both ring N atoms as well as the primary amino group are possible points of attachment. Since the thia diazole exchanges with the nicotinamide moiety of NAD in the presence of NADase, one might expect that the attachment would be to a ring nitrogen.

A point of some interest with respect to the structure of these derivatives is the marked differences in retention times between NAD and the NAD analog and between NMN and the thia diazole mononucleotide. These differences are similar to those reported by Ciotti et al. (7) for the relative electrophoretic migration and the retention on anion-ex-
change columns of NAD and their NAD analog containing 2-
ethylamino-1,3,4-thiadiazole. These authors suggested that their analog contained a tertiary nitrogen rather than a quaternary nitrogen. The behavior of our thia diazole deriva-
tives indicates that they also have a greater negative charge than do the corresponding nicotinamide derivatives. We are presently attempting chemical synthesis of a nucleotide of aminothiadiazole for comparison with both the metabolite isolated in vivo and the nucleotide formed by cleavage of the NAD analog.

The pathway for the formation of the thia diazole nucleo-
tide in vivo is also under investigation. Since the formation of the nucleotide is blocked by nicotinamide (Chart 6), a pathway involved in the metabolism of nicotinamide proba-
bly is responsible for its formation. Likely pathways are: Pathway a, reaction with phosphoribosylpyrophosphate in the presence of a phosphoribosyltransferase; Pathway b, the sequential action of a nucleoside phosphorylase and a nucleoside kinase; and Pathway c, formation of the NAD analog by exchange between the thia diazole and the nicotinamide moiety of NAD followed by cleavage of the NAD analog by nucleotide pyrophosphatase. Although we pre-
nared the nucleotide by Route c, this is probably not the route for its formation in vivo, because no NAD analog was found in L1210 cells and it is unlikely that the analog, if formed, would have been degraded completely to the thia-
diazole nucleotide. Mammalian cells are known to contain the enzymes required for formation of nicotinamide nucleo-
tide by Pathways a and b, namely, nicotinamide phosphor-
ibosyltransferase (8), nicotinamide ribonucleoside phospho-
ylase (15), and nicotinamide ribonucleoside kinase (14).

These pathways are shown in Chart 7.

A nucleotide of another 5-membered heterocycle also inhibits IMP dehydrogenase. Ribavirin (virazole), 1-(β-D-
ribofuranosyl)-1,2,4-thiazole-3-carboxamide, an antiviral agent, is converted to the phosphate which is a potent inhibitor, competitive with IMP (17).

The results reported here, together with earlier observations, indicate that the cytotoxicity and presumably the antitumor effects of aminothiadiazole and related com-
ounds are the result of inhibition of IMP dehydrogenase. It will be of interest to determine whether this blockade is also responsible for the remarkable uricogenic activity of the thia diazoles. A response of the cell to blockade of this enzyme might be an increased synthesis of purines de novo with a consequent accumulation of IMP which would be eliminated as uric acid. In accord with this mechanism is the observation that nicotinamide reversed both the antileu-
emic and the uricogenic activity of 2-ethylaminothia-
diazole (7, 11).

REFERENCES

2. Ames, B. N. Assay of Inorganic Phosphate, Total Phosphate and Phos-
6. Cha, S., Agarwai, R. P., and Parks, R. E., Jr. Tight-Binding Inhibitors—II. Non-Steady State Nature of Inhibition of Milk Xanthine Oxidase by Allo-
purinol and Alloxanthine and of Human Erythrocytic Adenosine Daami-
11. Krakoff, I. H., and Balis, M. E. Studies of the Uricogenic Effect of 2-

Chart 7. Pathways for the formation of nucleotide derivatives of amino-
thiadiazole. N, nicotinamide; T, aminothiadiazole; R, ribosyl; RP, 5-phos-
phoribosyl; P, phosphate, PRPP, phosphoribosylpyrophosphate; A, adenine.
Mechanism of Action of NSC 4728


Mechanism of Action of 2-Amino-1,3,4-thiadiazole (NSC 4728)

J. Arly Nelson, Lucy M. Rose and L. Lee Bennett, Jr.


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/1/182

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.