The Antileukemic Action of Two Thiadiazole Derivatives*

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SUMMARY

The thiadiazole derivatives 2-amino-1,3,4-thiadiazole and 2-ethylamino-1,3,4-
thiadiazole retarded the growth of leukemia L1210 and extended the survival time of
the mice. The thiadiazoles were approximately as effective as the folic acid antagonist
aminopterin but considerably less effective than amethopterin in increasing the life
span of the leukemic mice.

The antileukemic action and host toxicity of the thiadiazoles were blocked by ad-
ministration of nicotinamide.

In vitro exchange of thiadiazole with the nicotinamide moiety of DPN was ob-
tained, resulting in the formation of the thiadiazole analog of DPN. C14-labeled thiadia-
zole was readily incorporated in the various tissues of the leukemic host.

Preliminary attempts to demonstrate formation of the thiadiazole analog of DPN
in vitro were inconclusive.

It has been demonstrated previously (5) that
an exchange reaction may occur between the nic-
otinamide moiety of diphosphopyridine nucleotide
(DPN) and other pyridine compounds such as
acetylpyridine. This transglycosidase reaction is
catalyzed by animal tissue DPNases and results
in the formation of the corresponding analog of
DPN and free nicotinamide. It has been demon-
strated to occur in vivo and is particularly active
in neoplastic tissues (5). It has been suggested
(5) that the formation of coenzyme analogs might
be used as a basis for developing effective chem-
otherapeutic agents. In an attempt to use 3-acetyl-
pyridine as an antitumor agent, it was found that
with this compound therapeutic usefulness was
severely limited by toxicity for the host (5).

A series of 2-amino-1,3,4-thiadiazole derivatives
has been shown by Oleson et al. (6) to retard the
growth of a melanoma (S91), a glioblastoma

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(8110), and a lymphosarcoma (6C3HED) in mice. Of the analogs employed, 2-amino-1,3,4-thia-
diazole and 2-ethylamino-1,3,4-thiadiazole were the most effective. Shapiro et al. (7) demonstrated that
2-ethylamino-1,3,4-thiadiazole exerted a carcino-
static effect against Adenocarcinoma 755 in C57BL
mice. Oleson had observed that these compounds
behaved as nicotinamide antagonists, and Shapiro
et al. reported that the retardation of growth of
Adenocarcinoma 755 by 2-ethylamino-1,3,4-
thiadiazole could be blocked by the prior adminis-
tration of nicotinamide (7).

The above observations suggested study of the
antileukemic action of the thiadiazole derivatives
and investigation of the possibility that the thiadi-
zole compounds could be acting by forming the
thiadiazole analog of DPN. In preliminary reports
(1, 3) it was noted that 2-ethylamino-1,3,4-thia-
diazole and 2-amino-1,3,4-thiadiazole exerted a chemotherapeutic effect against mice bearing a
transplantable leukemia (L1210) and that the
antileukemic action could be blocked by nico-
tinamide. The current report details these findings.
Evidence is also presented that the thiadiazole
compounds exchange with nicotinamide in vitro,
resulting in the formation of thiadiazole analog of
DPN.

1195
MATERIALS AND METHODS

The general procedures have been described previously (2, 3). Eleven- to 13-week-old hybrid male mice (BALB/cAn × DBA/2J) were employed. Animals used in tumor studies were given inoculations in the right hind leg of 0.1 ml of a saline suspension of leukemic (L1210) cells prepared from the spleen of stock (DBA/2J) tumor-bearing mice. The leukemic cell suspensions employed in the various experiments contained from 5 to 10 million cells per ml. Treatments were initiated either early (3 days following tumor inoculation) or late (7-10 days following tumor inoculation) in the course of tumor growth and continued daily until the death of the animals. The mice were maintained on an ad libitum diet of Purina Laboratory Chow and water. There were ten animals in each experimental group and in each of the controls.

2-Amino-1,3,4-thiadiazole and nicotinamide were dissolved in water. 2-Ethylamino-1,3,4-thiadiazole-HCl, aminopterin, and amethopterin1 were dissolved in 2 per cent aqueous sodium bicarbonate.

Injections were given in the constant volume of 0.01 ml/gm of body weight except at the highest dose level of 2-amino-1,3,4-thiadiazole (500 mg/kg) for which a concentration of 25 mg/ml was given in the volume of 0.02 ml/gm of body weight. All drugs were administered subcutaneously, except nicotinamide, which was given intraperitoneally. When combination treatment was employed, nicotinamide was given immediately prior to the thiadiazole derivative.

Pig brain DPNase was employed to test for analog formation in vitro. The reaction mixture contained 2.05 ml of the enzyme, 210 μmoles of 2-ethylamino-1,3,4-thiadiazole-5-C14, 0.3 ml of 1.0 M potassium phosphate (pH 7.5), and 60 μmoles of DPN, brought to a final volume of 3 ml with water. An aliquot was removed prior to incubation for determination of radioactivity. The mixture was incubated at 37° C. Aliquots were assayed with yeast alcohol dehydrogenase until approximately 93 per cent of the DPN had been split. The reaction mixture was treated with trichloroacetic acid to a final concentration of 6 per cent, and the denatured protein was removed by centrifugation. The analog was then precipitated with 5 volumes of cold acetone and allowed to stand in the cold overnight. The precipitated analog was washed with acetone and then dissolved in water. Aliquots were plated for determination of radioactivity.

For the in vivo incorporation studies, 2-ethylamino-1,3,4-thiadiazole-5-C14 (500 mg/kg) was injected 7 days after leukemic inoculation. The tissues were removed 4 hours after injection and extracted with 5 volumes of 6 per cent trichloroacetic acid. Following centrifugation for 10 minutes at 4000 r.p.m., the samples were extracted with ether to remove the trichloroacetic acid. Aliquots (0.1 ml) were plated for determination of the radioactivity in the tissue extracts.

RESULTS

Antileukemic action.—Chart 1 shows that 2-amino-1,3,4-thiadiazole and 2-ethylamino-1,3,4-thiadiazole are both capable of increasing the life span of leukemic mice even when treatment is initiated late in the course of the disease. Both drugs increased the life expectancy of the mice approximately 5 times, as measured from the day of initiation of treatment. With both drugs, at the most effective dose levels, the local tumors at the site of leukemic inoculation showed marked regression, but this was accompanied by weight loss of the animals. Higher doses were more toxic for the host, and there was a resultant decrease in survival time.

As demonstrated in Chart 1, 2-ethylamino-1,3,4-thiadiazole was approximately as effective as aminopterin in prolonging survival time, but considerably less effective than amethopterin. Similarly, 2-amino-1,3,4-thiadiazole was considerably less effective than amethopterin.

The increase in survival time elicited by 2-ethylamino-1,3,4-thiadiazole against advanced leukemia was reversed by nicotinamide (Table 1). The reversal of antagonist action by nicotinamide was substantiated further by the results of two experiments in which treatment was initiated early in the course of the disease (Chart 2). When treatment with 2-ethylamino-1,3,4-thiadiazole or 2-amino-1,3,4-thiadiazole was initiated 3 days following inoculation of the leukemic cells (Chart 2), nicotinamide blocked the inhibitory effect of the thiadiazoles on the local tumor at the site of leukemic inoculation and prevented extension of the life span of the animals.

Table 2 shows that nicotinamide can block thiadiazole toxicity in nonleukemic mice. Five doses of 500 mg/kg and eleven doses of 250 mg/kg of 2-amino-1,3,4-thiadiazole were lethal for the mice. Protection against lethal toxicity at both dose levels was afforded by concomitant treatment with 500 mg/kg of nicotinamide. It is of interest to note that the nicotinamide did.

1 The drugs were kindly provided by the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, New York.
Chart 1.—Comparison of the relative effectiveness of 2-ethylamino-1,3,4-thiadiazole, 2-amino-1,3,4-thiadiazole, amethopterin, and aminopterin in increasing the life span of mice with advanced leukemia (LI210). In both experiments the controls were untreated. Part of these data is reported in Reference 3.

Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nicotinamide (mg/kg)</th>
<th>2-EAT (mg/kg)</th>
<th>Median S.T.* (days)</th>
<th>Av. tumor diameter (mm.) 10</th>
<th>Av. animal wt. change (gm.) 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1, treatment daily from day 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>500</td>
<td>250</td>
<td>18</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Exp. 2,† treatment daily from day 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>500</td>
<td>240</td>
<td>15</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>144</td>
<td>10</td>
<td>9.9 (9)</td>
<td>8.3 (9)</td>
<td>5.5 (2)</td>
<td>+0.8 (9)</td>
</tr>
<tr>
<td>80.4</td>
<td>10</td>
<td>10.1 (6)</td>
<td>7.4 (7)</td>
<td>5.5 (2)</td>
<td>+1.7 (6)</td>
</tr>
<tr>
<td>500</td>
<td>250</td>
<td>607</td>
<td>10</td>
<td>10</td>
<td>0.9 (9)</td>
</tr>
<tr>
<td>500</td>
<td>400</td>
<td>144</td>
<td>10</td>
<td>9</td>
<td>10.9 (6)</td>
</tr>
<tr>
<td>500</td>
<td>240</td>
<td>86.4</td>
<td>10</td>
<td>10.4 (5)</td>
<td>+2.7 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>500</td>
<td>86.4</td>
<td>10</td>
<td>10.8 (9)</td>
<td>+2.7 (4)</td>
</tr>
</tbody>
</table>

* S.T. = Survival time; day of inoculation of tumor is day 0.
† Part of the data of this experiment reported in Reference 3.
Ten animals per experimental group. Survivors, when less than ten, are indicated in parentheses.
CHART 2.—The antileukemic action and nicotinamide antagonism of 2-ethylamino-1,3,4-thiadiazole and 2-amino-1,3,4-thiadiazole when treatment was initiated early in the course of the disease. In the experiment with 2-ethylamino-1,3,4-thiadiazole (left panel), the control mice and the mice treated with the thiadiazole (without nicotinamide) were also given injections intraperitoneally of 0.01 ml. of a 1 per cent saline solution. The broken lines represent thiadiazole plus nicotinamide (500 mg/kg).

In the experiment with 2-amino-1,3,4-thiadiazole (right panel), the control mice and the mice treated with the thiadiazole (without nicotinamide) were also given injections intraperitoneally of 0.01 ml. of a 1 per cent saline solution. The broken lines represent thiadiazole plus nicotinamide (500 mg/kg).

TABLE 2
PROTECTION BY NICOTINAMIDE AGAINST THE TOXICITY OF 2-AMINO-1,3,4-THIADIAZOLE

<table>
<thead>
<tr>
<th>Nicotinamide</th>
<th>2-Amino-1,3,4-thiadiazole</th>
<th>Dead/total</th>
<th>Day 4</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>500</td>
<td>5/6</td>
<td>-2.6</td>
<td>-1.8</td>
<td>-0.2</td>
<td>+1.2</td>
</tr>
<tr>
<td>500</td>
<td>250</td>
<td>11/0/4*</td>
<td>+0.2</td>
<td>-0.8 (4)</td>
<td>+1.0 (4)</td>
<td>+1.0 (4)</td>
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<tr>
<td>500</td>
<td>125</td>
<td>15/0/6</td>
<td>+0.8</td>
<td>+1.0</td>
<td></td>
<td>+1.3</td>
</tr>
<tr>
<td>0</td>
<td>500</td>
<td>5/6/6</td>
<td>-3.8 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>250</td>
<td>11/4/4*</td>
<td>-1.5</td>
<td>-6.5 (4)</td>
<td>+1.3</td>
<td>+1.7</td>
</tr>
<tr>
<td>0</td>
<td>125</td>
<td>15/0/6</td>
<td>+1.3</td>
<td>+0.8 (4)</td>
<td>+1.5 (4)</td>
<td>+1.0 (4)</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>15/0/4*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Two of the six mice in these groups were sacrificed on the 9th day.
Av. wt. change calculated on six mice unless number is designated in parentheses.
not wholly prevent weight loss of the animals. This was most evident with the dose level of 500 mg/kg of 2-amino-1,3,4-thiadiazole.

Analog formation.—In preliminary in vitro studies of the exchange reaction with 2-ethylamino-1,3,4-thiadiazole it was not possible to isolate the coenzyme analog with certainty. This resulted from the difficulty in distinguishing the analog from adenosine-diphosphate-ribose. Both the absorption spectrum and the electrophoretic pattern of adenosine-diphosphate-ribose appeared to be similar to that of the thiadiazole analog. To obviate these difficulties, 2-ethylamino-1,3,4-thiadiazole-5-C\(^{14}\) was employed to study the formation of the thiadiazole analog of DPN.

An experiment showing the formation of the coenzyme analog in vitro is summarized in Table 3. In this reaction the DPNase from pig brain was used. To the incubation mixture was added 210 μmoles of the thiadiazole giving over 2 million counts per minute. Following incubation, coenzyme analog giving 63,000 counts per minute was obtained. From the 60 μmoles of DPN added, 5.9 μmoles of the DPN analog were formed, representing an exchange of 10 per cent—i.e., 10 per cent of the DPN was converted to the analog. It should be emphasized that this is a relatively low exchange as compared with the formation of the acetylpyridine analog from DPN, in which case almost all the DPN is converted to analog (5).

The thiadiazole analog of DPN can be distinguished from the natural coenzyme and from the free thiadiazole by paper electrophoresis or chromatography: (a) In Chart 3 the electrophoretic pattern of the analog is compared with DPN and the free thiadiazole. It may be noted that the free base moves toward the cathode, while DPN and the thiadiazole analog move toward the anode. As indicated, the thiadiazole analog of DPN moves farther than does the DPN. The DPN showed a net negative charge of 1, the analog a negative charge of 2. This might suggest that in contrast to DPN, which has a quaternary nitrogen, the thiadiazole analog of DPN has a tertiary nitrogen. (b) The thiadiazole analog can be isolated by chromatography on Dowex-1 formate. It requires much stronger acid (1.0 M formic acid-sodium formate) to remove the analog from the column than to remove the DPN. This is 10 times the concentration used for DPN separation. This again suggests that the analog is more negatively charged. (c) After acid and alkaline hydrolysis of the coenzyme analog, free thiadiazole was identified by paper chromatography. By the appearance of the free base it was determined that, whereas DPN is more stable in acid, the thiadiazole analog of DPN is more stable in alkali. (d) The 2-ethylamino-1,3,4-thiadiazole analog of DPN can be distinguished from DPN by its spectrum as shown in Chart 4. The analog has a broad peak with a maximum at 256 m\(\mu\), in contrast to the natural coenzyme, which has a sharper peak at 260 m\(\mu\).

Table 4 shows the distribution of radioactive label after a single injection of 500 mg/kg of 2-ethylamino-1,3,4-thiadiazole-5-C\(^{14}\) into mice bearing leukemia L1210. The level of radioactivity was roughly the same in the various tissues, including the local tumor at the site of leukemic inoculation. It is of interest to note that appreciable radioactivity was still present at 24 hours after injection of the thiadiazole derivative. Any formation of thiadiazole analog of DPN in vivo would appear to be of a low order.\(^2\)

\(^2\) Less than 5 per cent of the counts from liver were found in the nucleotide fraction associated with DPN.
DISCUSSION

The current studies demonstrate that 2-amino-1,3,4-thiadiazole and 2-ethylamino-1,3,4-thiadiazole inhibit the growth of leukemia L1210 and increase the survival time of the mice. The drugs are effective whether treatment is initiated early or late in the course of the disease. Although the limiting factor in the use of the drugs is the toxicity for the host, the antileukemic action does not appear to be attributable to nonspecific host toxicity. No degree of food or water restriction was capable of increasing the survival time of mice with advanced leukemia (4). Also, against both the early and late leukemia, increases in survival time were observed at doses which produced little or no animal weight loss, or only terminal weight loss.

It has been reported previously (7) that the aminothiadiazoles behave as nicotinamide antagonists with respect to solid tumors. The current experiments illustrate that the antileukemic action of the thiadiazoles can be blocked by the simultaneous administration of nicotinamide. This was evident when treatment was initiated either early or late in the course of the disease. That the block of antileukemic action of the thiadiazole represents a true metabolite-antimetabolite relationship is suggested by both the reversal of thiadiazole inhibition of local tumor growth by nicotinamide and the diminished survival time of the host. Evidence of nicotinamide antagonism is also provided by the observation that nicotinamide diminished the toxicity of the thiadiazoles for the host.

It had previously been demonstrated (5) that DPNases in animal tissues can catalyze an exchange reaction both in vitro and in vivo between the nicotinamide moiety of diphosphopyridine nucleotide (DPN) and compounds related to nicotinamide. Leukemia L1210 showed a relatively higher concentration of analog than other tissues after the administration of the nicotinamide antagonist 3-acetylpyridine. This antagonist lowered the concentration of DPN in leukemic tissue and partially blocked the synthesis of DPN from nicotinamide. In both cases, the decrease in DPN could be accounted for by the extent of analog formation.

Although some inhibition of local tumor growth could be demonstrated by administration of 3-acetylpyridine to leukemic mice, it was not possible to demonstrate appreciable increases in survival time of mice with advanced leukemia. It is possible that with 3-acetylpyridine the toxicity of the drug for the host is limiting and the animals succumb before sufficient formation of analog occurs in the tumor cells. The toxicity for the host may be traceable to rapid analog formation in brain tissue.

**Table 1**

| INJECTION OF 2-ETHYLAMINO-1,3,4-THIADIAZOLE-5-C\textsuperscript{14} INTO LEUKEMIC MICE |
|---|---|---|
| TISSUES | 4 hr. | 24 hr. |
| Kidney | 11,600 | 3,300 |
| Liver | 9,250 | 2,450 |
| Spleen | 7,850 | 2,000 |
| Muscle | 7,400 | 2,550 |
| Tumor | 6,950 | 2,150 |
| Brain | 8,050 | 3,800 |

2-Ethylamino-1,3,4-thiadiazole-5-C\textsuperscript{14} was dissolved in 2 per cent sodium bicarbonate and injected into each of six mice at a level of 500 mg/kg. Each mouse received about 1,150,000 counts/min of the labeled compound. After 4 and 24 hours, respectively, the mice were sacrificed and the tissues homogenized in 5 volumes of 6 per cent trichloroacetic acid. Aliquots were plated and then counted.
may inhibit DPN synthesis from nicotinamide (8). The current data demonstrate that in vitro the thiadiazoles will exchange with the nicotinamide moiety of DPN, resulting in the formation of the thiadiazole analog of DPN. Administration of the C14-labeled thiadiazole resulted in approximately equal distribution in the various tissues of the mouse, including the local tumor at the site of inoculation. Apparently, the compound is also capable of passing freely through the blood-brain barrier, as indicated by the incorporation in brain tissue. However, following single dose administration of the thiadiazole, the thiadiazole analog of DPN could not be clearly detected. No definite evidence could therefore be obtained on any possible relationship of analog formation to the in vivo action of thiadiazole. It should be emphasized, however, that, because the exchange reaction in vitro is of a relatively low order, this may be reflected in vivo. Since repeated doses of the thiadiazoles are required for toxicity and antitumor activity, multiple doses may be necessary to show coenzyme analog formation in vivo.

The availability of nicotinamide antagonists which inhibit tumor growth and engage in exchange reactions of coenzyme may provide a means for elucidating metabolic mechanisms and permit metabolic disturbances of processes vital for tumor growth.

REFERENCES


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