Prevention of Tubercidin Host Toxicity by Nitrobenzylthioinosine 5′-Monophosphate for the Treatment of Schistosomiasis

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Host toxicity of the dose regimen of tubercidin (7-deazaadenosine) plus nitrobenzylthioinosine 5′-monophosphate (NBMPR-P) used in combination therapy of schistosomiasis (M. H. el Kouni, D. Diop, and S. Cha, Proc. Natl. Acad. Sci. USA 80:6667–6670, 1983; M. H. el Kouni, N. J. Messier, and S. Cha, Biochem. Pharmacol. 36:3815–3821, 1987) was examined in vivo in mice and in vitro with human bone marrow progenitor cells. Four successive daily intraperitoneal injections of tubercidin at 5 mg/kg per day produced 100% mortality in mice within 3 to 5 days following the first injection, with massive peritonitis and intestinal obstruction secondary to abdominal adhesions. Coadministration of NBMPR-P (25 mg/kg per day) protected the mice from the lethality of tubercidin and allowed the repetition of the regimen for a second time with 100% survival until the mice were sacrificed 22 days following the first injection. Blood chemistry, hematological studies, and histological examinations showed no evidence for injury to the liver, kidney, spleen, pancreas, mesentery, or peritoneal mesothelium. In vitro, tubercidin alone had a direct dose-dependent inhibitory effect on myeloid and erythroid human bone marrow progenitor cells, and consistent inhibition (50%) of granulocyte-macrophage CFU (CFU-GM) and erythroid burst-forming units (BFU-E) occurred at 2 to 3 nM tubercidin. At higher doses, BFU-E were more sensitive to tubercidin toxicity than CFU-GM. Complete inhibition (99%) of BFU-E colonies occurred at 10 nM tubercidin, while complete inhibition of CFU-GM occurred at 100 nM. NBMPR-P at 10 to 100 nM protected CFU-GM and BFU-E from tubercidin toxicity in a dose-dependent manner.

Tubercidin (7-deazaadenosine) is the most effective anti-schistosomal purine nucleoside analog tested to date (14, 22). Unfortunately, attempts to use tubercidin in vivo have been hampered by severe host toxicity (5–7, 14). Recently, however, it was demonstrated that nitrobenzylthioinosine 5′-monophosphate (NBMPR-P), a produg of the potent nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR) (21) did not prevent the uptake of tubercidin by Schistosoma mansoni, in contrast to its effects on mammalian systems (4, 5). Therefore, when NBMPR-P was coadministered with tubercidin to S. mansoni- and Schistosoma japonicum-infected mice, tubercidin was made selectively toxic against the parasites by protection of the host from tubercidin toxicity (5–7). The combination of tubercidin plus NBMPR-P was also effective against trypanosomiasis (19) and malaria (9, 10). Nevertheless, there have been reservations about using this combination, primarily because of the known severe toxicity of tubercidin alone, which stems mainly from hepatic and renal damage (3, 15, 16, 18, 23, 24). NBMPR is neither lethal nor toxic at the doses used in the present study (5–7, 16, 17). In contrast to the extensive studies on the toxicity of tubercidin and NBMPR-P as single drugs (3, 15–18, 23, 24), the pharmacology and pathology of the combination of the two drugs was only studied as a single-dose regimen (16). No information is available on the toxicity of extended exposure to the combination of these drugs as required for the successful treatment of schistosomiasis, except that NBMPR-P protected the host from the lethality of tubercidin (5–7).

Therefore, in the present investigation, in addition to monitoring survivorship, we performed histological, biochemical, and hematological studies in mice to evaluate the in vivo toxicity of the dose regimen of tubercidin plus NBMPR-P used in combination therapy of schistosomiasis. We also investigated the in vitro toxicity of tubercidin, NBMPR-P, and the combination of tubercidin plus NBMPR-P on human myeloid and erythroid colony-forming cells since host bone marrow cells are particularly sensitive to nucleoside analogs and little is known about the effect of the two drugs on these cells.

MATERIALS AND METHODS

Chemicals. Tubercidin was purchased from Sigma Chemical Co. (St. Louis, Mo.), and NBMPR-P was provided by A. R. P. Paterson (Cancer Research Unit, McEachern Laboratory, University of Alberta, Edmonton, Alberta, Canada).

Toxicity studies in vivo. Three groups of mice were used, each consisting of 35 female (20- to 25-g) CD1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The first group received four successive daily intraperitoneal injections of tubercidin (5 mg/kg per dose) plus NBMPR-P (25 mg/kg per dose) (17); the regimen was repeated after a 10-day rest period (5–7). The second group received four daily injections of tubercidin (5 mg/kg per dose) alone, but the regimen could not be repeated owing to the death of mice within 3 to 5 days of the initiation of treatment. The third group (controls) received saline solution (0.9% NaCl). Drugs were prepared and administered as previously described (5–7). All mice that survived were sacrificed 4 days after the last injection. Whole blood was obtained by cardiac puncture and collected in a Unopette microcollection system (Becton
Dickinson and Co., Paramus, N.J.), and a complete blood count was determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). For blood serum chemist-

ry, sera were obtained by centrifugation of whole blood and analyzed by standard methodologies for the levels of blood urea nitrogen, serum glutamic oxalacetic transaminase, se-

rum glutamic pyruvic transaminase, alkaline phosphatase, creatinine, glucose, amylase, bilirubin, and lactate dehydro-

genase. For histological examination, tissue samples from

sacriﬁced mice were ﬁxed in 10% buffered Formalin acetate

(pH 7.0 to 7.1) and stored at 4°C until section preparation.

Tissues were embedded in parafﬁn, sectioned at 5 µm, and

stained with hematoxylin and eosin.

Toxicity studies with human bone marrow progenitor cells

in vitro. Human bone marrow cells were obtained from healthy volunteers, and mononuclear cells were isolated as

previously described (25). Viability was >98% as assessed by

the trypan blue exclusion method. A bilayer soft agar

method was used to culture granulocyte-macrophage CFU

(CFU-GM) (25). Portions of 1 ml of the top layer containing

the mononuclear nonadherent cells (10

6 cells per ml) were

plated in 35-mm plastic petri dishes on a 1-ml bottom layer

containing the desired drug concentration. For the erythroid

burst-forming unit (BFU-E) assays (25), 1 ml of mononuclear

cells (10

5 cells per ml) in methylcellulose was plated in petri

dishes containing 0.1-ml solutions of the drug(s). In the

protection studies, cells were exposed to 10 nM of tubercidi-

n (70% inhibitory concentration [IC

50] and IC

90 for CFU-GM

and BFU-E, respectively) in the presence or absence of

various doses of NBMPR-P. Cells were continuously ex-

posed to drugs for 14 days in a humidified atmosphere of 5%

CO

2–95% air at 37°C. Colonies of CFU-GM (≥50 cells) and

BFU-E (≥30 hemoglobinized cells) were counted with an

inverted microscope.

RESULTS

Survivorship. All animals receiving tubercidin alone died

between days 3 and 5 after initiation of treatment, and a

second regimen could not be given. The dead mice exhibited

massive peritonitis and intestinal obstruction secondary to

abdominal adhesions. Co-administration of NBMPR-P with

tubercidin resulted in complete protection from the lethality

of tubercidin and allowed the repetition of the regimen for a

second time, with 100% of the mice surviving until they were

sacriﬁced 22 days after the ﬁrst injection.

Histological examination. Examination of the intestines

from treated mice showed well-preserved mucosa without

necrosis, ulceration, or inﬂammation. By light microscopy,

the kidneys were unaffected by this combination chemotherapy. There was no glomerulonephritis or basement membrane thickening. The tubules were intact, without signs of necrosis. There was no interstitial nephritis. No changes in the liver were observed. Speciﬁcally, there was no fatty change or necrosis. There was no difference between the spleens from control and treated mice. The pancreas of treated mice was without inﬂammation or necrotic areas.

Hematological and serum biochemical analyses. Tables 1

and 2 show no signiﬁcant differences between the treated

group and their respective controls in any of the indices

studied.

Studies on normal human bone marrow progenitor cells.

Continuous exposure for 14 days to tubercidin alone was

highly toxic to both human CFU-GM and BFU-E (Fig. 1 and

2). The IC

50 of tubercidin were 3.4 ± 1.7 and 3.7 ± 0.2 nM

for CFU-GM and BFU-E, respectively. However, the IC

90 for BFU-E (8.6 ± 0.2 nM) was lower by 1 order of magnitude

than that for CFU-GM (71.2 ± 8.9 nM). In contrast, NBMPR-P at 100 nM was not toxic to either CFU-GM or BFU-E. Even at the higher concentration of 500 nM NBMPR-P, 60% of CFU-GM and BFU-E colonies were still

TABLE 1. Effect of combination therapy with tubercidin

(5 mg/kg) plus NBMPR-P (25 mg/kg) on hematological indices in mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline controls</th>
<th>Tubercidin + NBMPR-P</th>
</tr>
</thead>
</table>
| Leukocyte count (10

9/µl) | 2.7 ± 0.7       | 2.4 ± 0.4            |
| Erythrocyte count (10

12/µl) | 6.2 ± 0.4       | 6.7 ± 1.4            |
| Hemoglobin (g/dl)         | 13.1 ± 0.7      | 13.3 ± 2.6           |
| Hematocrit (%)            | 32.8 ± 2.2      | 34.7 ± 7.7           |
| Mean corpuscular vol (fl) | 53.0 ± 1.6      | 51.3 ± 1.4           |

* The combination of tubercidin plus NBMPR-P was administered by four successive daily intraperitoneal injections. The regimen was repeated after a 10-day rest period. Saline controls received saline only. Mice were sacriﬁced 4 days after the last injection.

* Mean ± SD of values obtained from at least nine different mice.

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TABLE 2. Effect of combination therapy with tubercidin

(5 mg/kg) plus NBMPR-P (25 mg/kg) on the level of serum enzymes in mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline controls</th>
<th>Tubercidin + NBMPR-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/liter)</td>
<td>170 ± 21</td>
<td>158 ± 6</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/liter)</td>
<td>24 ± 0</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Creatinine (mg/liter)</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Bilirubin (mg/liter)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>SGOT* (IU/liter)</td>
<td>106 ± 29</td>
<td>160 ± 22</td>
</tr>
<tr>
<td>SGPT† (IU)</td>
<td>25 ± 0.8</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Lactate dehydrogenase (IU/liter)</td>
<td>1,183 ± 245</td>
<td>1,488 ± 128</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/liter)</td>
<td>88 ± 12</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>Amylase (IU/liter)</td>
<td>1,500 ± 170</td>
<td>1,676 ± 163</td>
</tr>
</tbody>
</table>

* The combination of tubercidin plus NBMPR-P was administered by four successive daily i.p. injections. The regimen was repeated after a 10-day rest period. Saline controls received saline only. Mice were sacriﬁced 4 days after the last injection.

† Mean ± SD of values obtained from sera pooled from two groups of 10 mice each.

‡ SGOT, serum glutamic oxalacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

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FIG. 1. Effects of continuous exposure (14 days) to increasing concentrations of tubercidin or NBMPR-P on colony formation of human CFU-GM grown in soft agar compared with controls. Each point represents the mean ± standard deviation (SD) of at least six experiments with different marrow donors.
viable (Fig. 1 and 2). Furthermore, NBMPR-P at 10 to 100 nM clearly protected CFU-GM and BFU-E colonies against the toxicity of 10 nM tubercidin in a dose-dependent manner (Fig. 3). NBMPR-P at 50 nM or higher caused an increase in the number of CFU-GM colonies from 30 to 66% compared with controls. Similarly, the presence of NBMPR-P at 10 and 50 nM increased the number of BFU-E colonies from 1% to 26 and 47%, respectively, compared with controls.

**DISCUSSION**

The present results clearly demonstrate that tubercidin at the dose regimen used in this investigation (5 mg/kg every day for 4 days) is lethal when used alone. It produced 100% mortality within 3 to 5 days after the first injection. Previous toxicological studies showed that the mortality from tubercidin is primarily due to hepatic and renal injuries and, to a lesser extent, damage to the pancreas. Generalized lymphoid depression, pyknosis of lymphocytes, reticulocytopenia, and slight reduction in cellularity of the bone marrow were also observed (3, 15, 16, 18, 23, 24). Coadministration of NBMPR-P at 25 mg/kg per day completely protected the mice (100% survival) from this lethal dose regimen of tubercidin. NBMPR-P can extend the life span of mice not only to the 22 days obtained in the present study but beyond 17 weeks after the first injection (unpublished data). The protection of the mice from tubercidin toxicity by NBMPR-P is presumably mediated by the property of NBMPR as a potent nucleoside transport inhibitor. It inhibits the uptake and subsequently the toxicity of tubercidin as well as other nucleoside analogs in many mammalian cells (21).

Contrary to what was observed when tubercidin was administered alone (3, 15, 16, 18, 23, 24), there was no evidence for injury to the liver, pancreas, mesentery, or peritoneal mesothelium when NBMPR-P was coadministered with NBMPR-P. NBMPR-P protects these organs from tubercidin toxicity by substantially decreasing their uptake of the drug (16). The lack of renal damage in the present results disagrees with the findings of Kolassa et al. (16). This is probably because the dose of tubercidin (5 mg/kg) we used in combination with NBMPR-P constitutes less than two-thirds of its 50% lethal dose in the presence of 25 mg/kg of NBMPR-P (17), while Kolassa et al. (16) used higher doses of tubercidin (15 mg/kg) and NBMPR-P (100 mg/kg). Furthermore, the renal damage caused by this combination was due indirectly to the high dose of NBMPR-P (100 mg/kg), since treatment with this dose of NBMPR-P increased the distribution of tubercidin in the kidney (16).

The present results also show that tubercidin has a direct dose-dependent inhibitory effect on myeloid and erythroid human bone marrow progenitor cells in vitro. Consistent inhibition (50%) of CFU-GM and BFU-E occurred with 2 to 3 nM tubercidin. At higher doses, BFU-E were more sensitive to tubercidin toxicity than CFU-GM. Complete inhibition (99%) of BFU-E colonies occurred at 10 nM tubercidin, while complete inhibition of CFU-GM occurred at 100 nM. NBMPR-P at nontoxic doses (10 to 100 nM) protected CFU-GM and BFU-E from tubercidin toxicity. The percent protection depended on the concentration of NBMPR-P and the degree of tubercidin toxicity, suggesting that protection is a competitive process and consistent with the modulation of tubercidin uptake by NBMPR-P. Although NBMPR-P did not protect the cells completely (100%) from tubercidin toxicity, it should be noted that these are long-term continuous exposure experiments (2 weeks). In a clinical situation, better results may be expected since the half-life of tubercidin is approximately 10 s and in the presence of NBMPR-P this value is only extended to 26 s (16). Therefore, it is reassuring that NBMPR-P can afford 6,700-fold-longer duration of protection against tubercidin toxicity (2 weeks versus 180 s = six times the half-life of about 30 s). It is also reassuring that protection of human bone marrow progenitor cells from tubercidin toxicity by NBMPR-P was also observed in short-term (1-h) exposure experiments in which NBMPR-P increased the IC_{50} of tubercidin by 10- to 100-fold (A. Janowska-Weiczorek and C. E. Cass, Am. Assoc. Cancer Res. 28:409, 1987).

In conclusion, previous (4-7, 9, 16, 17, 19) and present results demonstrate that tubercidin toxicity of the host can be prevented with a high degree of selectivity by NBMPR-P. The usefulness of such a regimen is not limited to the treatment of schistosomiasis or restricted to these particular two drugs. Malaria (9, 10) and trypanosomiasis (19) were treated successfully with tubercidin plus NBMPR-P. Combinations of other nucleoside analogs and nucleoside transport inhibitors were also shown to be effective against schistosomiasis (7) and malaria (10). As in schistosomes (4, 5), NBMPR and other nucleoside transport inhibitors do not inhibit the uptake of tubercidin in Trypanosoma gambiensc (19, 20) and Plasmodium yoelii (9) or Plasmodium falci-
parum (10)-infected erythrocytes as opposed to their effect on noninfected host cells. If this insensitivity to inhibitors of nucleoside transport in mammalian cells is a common property of nucleoside transport among parasites or parasite-infected cells, as so far appears to be the case (1, 4, 5, 9–11, 20), this mode of host protection by combination chemotherapy may provide an attractive alternative for the treatment of parasitic diseases with currently available antiparasitic cytotoxic nucleoside analogs (2, 4, 8–10, 12–14, 19, 22).

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LITERATURE CITED