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MANIPULATION OF TOXICITY AND TISSUE DISTRIBUTION OF TUBERCIDIN IN MICE BY NITROBENZYLTHIOINOSINE 5′-MONOPHOSPHATE*

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Abstract—The i.v. administration of tubercidin, an analog of adenosine, in a single dose of 45 mg/kg caused death in about 90% of B10D2F$_1$ mice so treated. Serum and urine analysis, as well as histological examination of tissues, related the lethality of tubercidin to hepatic injury, which was markedly reduced when mice were treated with the inhibitor of nucleoside transport, nitrobenzylthioinosine 5′-monophosphate (NBMPR-P), at i.p. doses higher than 10 mg/kg 30 min prior to tubercidin injection. With high NBMPR-P doses (100 mg/kg, i.p.) followed by tubercidin injection (45 mg/kg, i.v.), kidney damage and high mortality occurred. The tissue distribution of ^{3}H following [G-^{3}H]tubercidin administration paralleled hepatic or renal injury: NBMPR-P treatment decreased the content of tubercidin-derived ^{3}H in liver and increased that in kidney. Furthermore, the half-life of the decline in tubercidin levels in serum during the first minute after [^{3}H]tubercidin administration was longer in NBMPR-P-treated mice (26 sec) than in untreated mice (10 sec), with the result that ^{3}H levels in serum were more than ten times higher in the former than in the latter at an early stage during the distribution of tubercidin. Within 15 min after i.p. administration, the tissue distribution of [^{3}H]tubercidin was complete. The i.p. administration of tubercidin caused ascites and the appearance of amylase in the peritoneal fluid evidently because of peritonitis and pancreatic injury. Administration of NBMPR-P by the i.p. route, but not by the i.v. route, prevented these injuries and shifted the LD_{50} of i.p. injected tubercidin (5 mg/kg) to markedly higher values (a 4-fold increase with NBMPR-P at 100 mg/kg). The protection of mice by NBMPR-P against lethal injuries caused by i.p. injected tubercidin was consistent with the inhibition by NBMPR-P of tubercidin accumulation in mesentery and pancreas. The tissue specificity of the NBMPR-P influence on the tissue distribution of tubercidin may reflect differences in NBMPR-P pharmacokinetics and/or in properties of the nucleoside permeation mechanism among various tissues.

Earlier reports from this laboratory showed that neoplastic cells proliferating in culture medium containing NBMPR‖ were protected by the latter against the antiproliferative effects of tubercidin¶ [1, 2]. Reduction in the transporter-mediated entry of tubercidin into cells was the apparent basis of the NBMPR protective effect, since NBMPR is a potent, tightly bound inhibitor of nucleoside transport [3]. Tubercidin and adenosine are competitive substrates for the nucleoside transport mechanism of L5178Y mouse lymphoma cells [4]. Intracellular phosphorylation of tubercidin by adenosine kinase [5–7] is probably not influenced by NBMPR [8–10]. Phosphorylation of intracellular tubercidin is an essential step in the manifestation of tubercidin cytotoxicity [11, 12].

Our supposition that NBMPR might decrease the uptake of tubercidin into vital tissues *in vivo* was tested by measuring the influence of NBMPR on tubercidin toxicity in mice; these experiments showed that a potentially lethal treatment regimen with tubercidin was tolerated by mice when NBMPR and tubercidin were administered simultaneously by the i.p. route [13, 14]. Similar findings have been reported with respect to protection of mice by NBMPR against the toxic nucleosides, nebularine and toyocamycin [13], and against a potentially lethal treatment schedule consisting of 3-deazauridine and 1-β-D-arabinofuranosylcytosine [15].

NBMPR-P, the 5′-monophosphoester of NBMPR, has been employed in *in vivo* experiments as a readily soluble form of NBMPR. NBMPR-P *per se* does not inhibit nucleoside transport, but NBMPR released by dephosphorylation is found associated with NBMPR-P-treated cells [16] and is responsible

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‖ Abbreviations: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBMPR-P, NBMPR 5′-monophosphate; HNBTGR (hydroxynitrobenzylthioguanosine), 2-amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; BUN, blood (serum) urea nitrogen; GPT, glutamate pyruvate transaminase; AP, alkaline phosphatase; i.v., intravenous; i.p., intraperitoneal; and p.o., peroral.

¶ Tubercidin, 7-deazaadenosine or 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine.

for the reported inhibition of nucleoside uptake by NBMPR-P [17]. We have reported that mice were protected against potentially lethal doses of nebularine when NBMPR-P was administered with the toxic nucleoside [13, 18].

The present study attempted to relate the influence of NBMPR-P on lethality of single doses of tubercidin (i.v. or i.p.) to (i) changes in tissue distribution of tubercidin, and (ii) changes in a number of variables, histological and biochemical, which reflected tissue-specific injury resulting from tubercidin administration.

MATERIALS AND METHODS

[G-^{3}H]Tubercidin was purchased from Moravek Biochemicals, Brea, CA, and [*carboxyl*-^{14}C]inulin from ICN Pharmaceuticals, Inc., Irvine, CA. Tubercidin was provided by the Upjohn Co., Kalamazoo, MI, through the courtesy of Dr. G. L. Neil. HNBTGR [19] and disodium NBMPR-P [18] were synthesized in this laboratory starting with 6-thioinosine provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

B10D2F$_1$ mice (C57BL/10J × DBA/2J, F$_1$), 20–30 g were obtained from the Health Sciences Small Animal Program, University of Alberta. Drugs were dissolved in 0.15 M NaCl and administered in volumes proportional to (i) 20 ml/kg body weight (tubercidin alone or in combination with NBMPR-P), or (ii) 10 ml/kg body weight (NBMPR-P). These agents were administered i.p., or i.v. into tail veins, and deaths were recorded daily. Urine content of glucose, ketones, blood, proteins and bilirubin was determined in a semi-quantitative manner by use of reagent strips (Labstix and Icotest, Ames Co. Div., Miles Laboratories, Rexdale, Ontario, Canada). Animals were decapitated for analysis of serum and peritoneal fluid and for histological examination of various tissues. Levels of amylase in peritoneal fluid, and of glucose, amylase, BUN, creatinine, bilirubin (total), GPT and AP in serum were determined* by standard procedures. For histological examination, tissue samples were obtained 4 days after treatment of mice with toxic doses of tubercidin and were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 5 μ, and stained with hematoxylin and eosin. In all instances, comparisons were obtained between corresponding tissue sections from treated and untreated mice.

In experiments which assessed the *in vivo* distribution of tubercidin, injected doses contained 20 μCi [^{3}H]tubercidin per mouse. Mice that had been starved for 18 hr were employed. For urine collection, mice were kept in beakers on filter paper for intervals of up to 4 hr, or in metabolism cages for 24-hr sampling periods. Animals were decapitated to obtain blood and tissue samples; the latter were weighed and dried overnight at 100°. The ^{3}H content of all samples was measured by liquid scintillation counting after combustion in a Packard model 306 Sample Oxidizer.

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RESULTS

Protection of mice by NBMPR-P against the toxicity of i.v. administered tubercidin. Single doses of i.v. administered tubercidin caused death in (i) about 50% of B10D2F$_1$ mice at a dose of 35 mg/kg body weight (LD_{50}), and (ii) about 90% of mice treated with 45 mg/kg (LD_{90}). Mean survival times were about 11 days. When the dose of tubercidin was 30 mg/kg, no deaths occurred during the 30-day observation period, whereas at 50 mg/kg all mice died within 7 days (Fig. 1).

Prior treatment of mice with NBMPR-P reduced the toxicity of i.v. administered tubercidin. As pretreatment doses of NBMPR-P were increased to 18 mg/kg, mortality in mice that received a single injection of tubercidin at the LD_{90} level (45 mg/kg) decreased to less than 20%. As doses of NBMPR-P were increased above 25 mg/kg, the protective effect was reduced and was not apparent at 100 mg/kg. The administration of NBMPR-P by the i.p. route was somewhat more effective in protecting mice aginst tubercidin lethality than NBMPR-P given by the i.v. route (Fig. 1).

Urine from mice treated i.v. with tubercidin at 45 mg/kg contained high concentrations of bilirubin and, at necropsy, jaundice was clearly evident in these mice. When mice were treated by either route with NBMPR-P at 100 mg/kg 30 min prior to receiving i.v. tubercidin at 45 mg/kg, mortality was not reduced relative to that from tubercidin alone, but the signs of liver injury (bilirubin and jaundice) were absent. Mice that received the combined treatment showed signs of kidney malfunction in that urine samples contained blood, glucose and high concentrations of protein (data not shown).

A 9-fold increase was observed in the serum glutamate-pyruvate transaminase (GPT) activity of mice which had received i.v. tubercidin (30 mg/kg) 4 days prior to sampling (Fig. 2). This result reflected minor liver damage without associated mortality

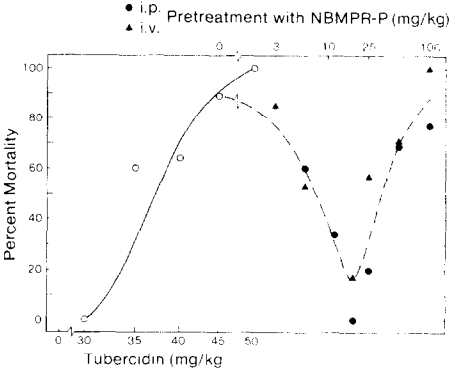


Fig. 1. Protection of mice by NBMPR-P against the lethality of i.v. administered tubercidin. Male B10D2F$_1$ mice received a single i.v. injection of tubercidin (○) at doses specified on the lower abscissa scale, or received NBMPR-P [by the i.p. (●) or i.v. (▲) routes] at doses specified on the upper abscissa scale 30 min prior to the i.v. administration of tubercidin at 45 mg/kg. Deaths were recorded daily for the next 30 days. The data represent the mortality seen in groups of six to fifty-four mice.

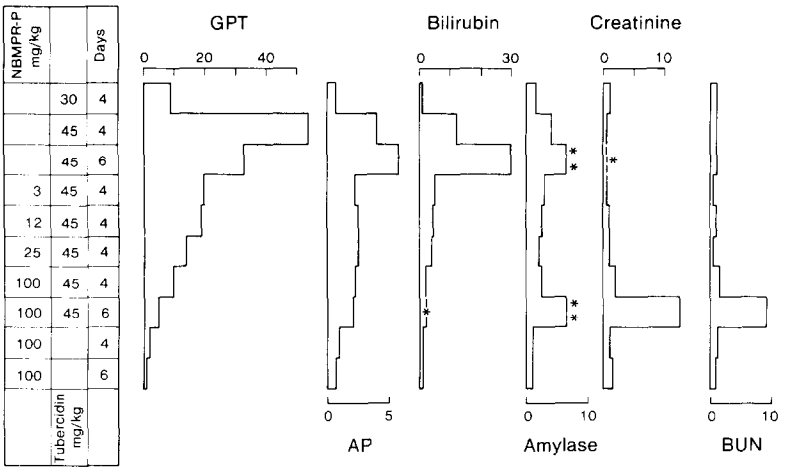


Fig. 2. Influence of prior treatment with NBMPR-P on biochemical variables in serum of mice treated i.v. with tubercidin. Male B10D2F$_1$ mice received NBMPR-P by i.p. injection 30 min prior to the i.v. injection of tubercidin and, at specified times thereafter, were decapitated for blood sampling. Serum samples for analysis were obtained from pooled blood samples from eight mice which had received identical treatment. Values for the several biochemical variables assayed in serum from treated mice (one or two determinations) are expressed as multiples of the corresponding values in serum from untreated animals [means (N = 3–8) ± S.D.]: GPT, 58 ± 36 I.U./l; AP, 112 ± 24 I.U./l; total bilirubin, 4 ± 1 mg/l; amylase, 1220 ± 10 I.U./l; creatinine, 3 ± 1 mg/l; BUN, 280 ± 30 mg/l. Asterisks: not done (*); content of serum sample exceeded the upper limit of the assay (**).

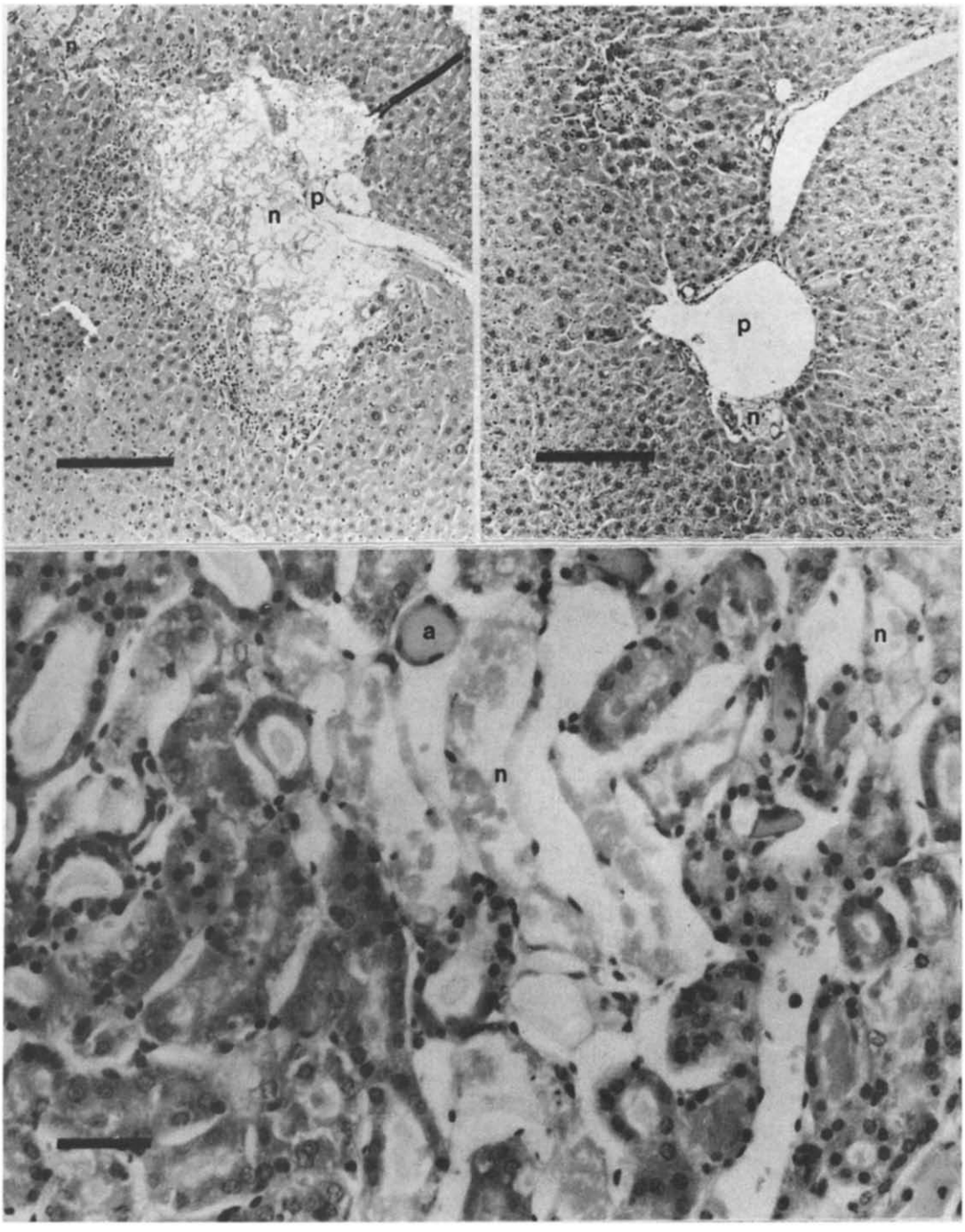
(serum AP activities were within the range of values obtained with untreated mice and serum bilirubin levels were only slightly elevated). Four days after the i.v. administration of tubercidin at 45 mg/kg (LD_{90}), serum GPT activities were increased more than 50-fold above control values. Similarly, AP activities and bilirubin concentrations in serum were elevated significantly on day 4 and were further elevated on day 6 after drug injection. The liver damage apparent in these data probably contributed to the mortality that resulted from the administration of tubercidin. The more than 4-fold rise in serum amylase activity that followed treatment with i.v. tubercidin at 45 mg/kg most likely reflected damage to pancreatic acinar cells. Creatinine and BUN levels showed no elevation in the serum of mice treated with tubercidin at this dose (LD_{90}), suggesting that renal function was not impaired. Significant changes in serum glucose levels did not occur in these experiments (data not shown).

The pretreatment of mice with NBMPR-P prevented the increases in serum levels of GPT, AP and bilirubin which otherwise followed the i.v. administration of tubercidin at 45 mg/kg; this effect was dependent on the NBMPR-P dose (Fig. 2). The minor increases in these variables observed when treatment with NBMPR-P at 100 mg/kg preceded tubercidin at 45 mg/kg were similar to those which occurred after the non-lethal dose of tubercidin at 30 mg/kg. These results indicate that NBMPR-P pretreatment protected mice aginst tubercidin-induced liver damage. This protection of liver may be part of the tolerance of NBMPR-P-treated mice toward potentially lethal doses of tubercidin (Fig. 1).

Figure 2 also shows that the tubercidin-induced increase in serum amylase levels was reduced by prior administration of NBMPR-P, suggesting partial protection against pancreatic injury.

Treatment of mice with 100 mg/kg NBMPR-P prior to the i.v. administration of tubercidin at 45 mg/kg resulted in a slight rise in serum levels of creatinine and BUN 4 days after drug injections (Fig. 2). Two days later, creatinine and BUN concentrations in serum exceeded those in untreated mice by about 10-fold. No significant increase in these variables occurred when either of the drugs was administered alone. These data suggest that impairment in kidney function (i) resulted from administration of tubercidin in combination with high doses of NBMPR-P, and (ii) may have contributed to the mortality observed when the NBMPR-P dose was raised beyond that eliciting the best protective effect (18 mg/kg NBMPR-P pretreatment; see Fig. 1).

These biochemical indications of drug injury in particular tissues were confirmed by histological findings. Microscopic examination of sections from livers of moribund mice which had been treated each with a single i.v. dose of tubercidin at 45 mg/kg (LD_{90}) showed extensive drug injury, with areas of necrosis around the portal veins (Fig. 3). Treatment of mice with protective doses of NBMPR-P (25 mg/kg) prior to otherwise toxic doses of NBMPR-P (45 mg/kg) resulted in mild hepatitis and small foci of hepatic necrosis (Fig. 4). Histological examination also revealed renal edema and some tubular necrosis in these animals. However, treatment of mice with high doses of NBMPR-P (100 mg/kg) prior to potentially lethal doses of tubercidin (45 mg/kg) produced severe renal tubular necrosis (Fig. 5). Liver damage in the latter animals was mild, being limited to small foci of inflammation or necrosis which did not extend beyond the portal areas. Renal or hepatic histopathological effects were not seen as a consequence of the i.p. administration of NBMPR-P as a single agent at 100 mg/kg.



Figs. 3–5. (Upper left panel—Fig. 3): Hematoxylin-eosin stained section of liver from a mouse 4 days after the i.v. administration of tubercidin at 45 mg/kg. Lymphocytic infiltration borders an area of necrosis (n) around a portal vein (p). Scale = 0.2 mm. (Upper right panel—Fig. 4): Hematoxylin-eosin stained section of liver from a mouse 4 days after the administration of NBMPR-P (25 mg/kg) by the i.p. route, followed 30 min later by i.v. injection of tubercidin (45 mg/kg). Periportal necrosis and lymphocytic infiltration are reduced relative to Fig. 3. Scale = 0.2 mm. (Bottom panel—Fig. 5): Hematoxylin-eosin section of kidney from a mouse 4 days after the i.p. injection of NBMPR-P (100 mg/kg) followed 30 min later by the i.v. administration of tubercidin (45 mg/kg). Apparent are extensive tubular necrosis (n) and distended tubules containing amyloid (a). Scale = 0.2 mm.

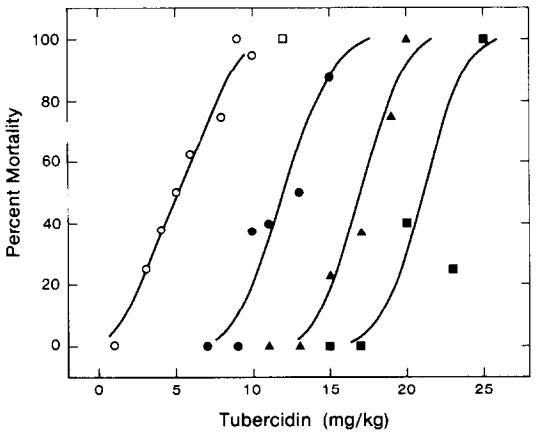


Fig. 6. Protection of mice by NBMPR-P against the lethality of single doses of i.p. administered tubercidin. Male B10D2F$_1$ mice in groups of eight were treated by the i.p. route with tubercidin administered alone (○) at the doses indicated, or simultaneously with NBMPR-P, the latter at these doses (mg/kg): 2 (●), 25 (▲), and 100 (■). Deaths were recorded daily for the next 30 days.

Protection of mice by NBMPR-P against the toxicity of i.p. administered tubercidin. Dose–mortality relationships in mice treated with single i.p. doses of tubercidin were explored in the experiment of Fig. 6. Mice that received single i.p. doses of tubercidin at 1 mg/kg survived the 30-day observation period, and all mice died following the i.p. injection of tubercidin at 10 mg/kg. The LD_{50} was about 5 mg/kg. Tubercidin was more toxic when administered by the i.p. route than by i.v. injection (compare Figs. 6 and 1). The simultaneous administration of NBMPR-P reduced the toxicity of i.p. administered tubercidin to the extent that LD_{50} values for tubercidin were increased to 12, 17 and 21 mg/kg by the co-administration of NBMPR-P at 2, 25 and 100 mg/kg respectively.

The modulating effect of NBMPR-P on the toxicity of tubercidin was diminished when the two agents were administered separately by the i.p. route. Both the sequence of the agents and the time interval between their separate injections were determinants of effect. When mice (in groups of eight) were treated with NBMPR-P at 50 mg/kg, administered together with, or 30 min prior to, tubercidin (15 mg/kg), only one mouse died, whereas all mice died within 5 days following treatment with tubercidin alone at 15 mg/kg (Table 1). When the injection of NBMPR-P followed that of tubercidin, the NBMPR-P protective effect was reduced markedly. For example, 25% mortality was observed when NBMPR-P was given 2 min after tubercidin and, when time intervals between administration of tubercidin and NBMPR-P were increased further, protection against tubercidin lethality was not achieved although some increase in life-span did result.

The lethality of single i.p. doses of tubercidin at 15 mg/kg was prevented by prior treatment of mice with i.p. NBMPR-P at 100 mg/kg. It is seen in Table 2 that the protective effect of NBMPR-P was dose dependent and was absent at i.p. doses of 6 mg/kg. Pretreatment with NBMPR-P by the i.v. route at doses as high as 100 mg/kg did not protect mice against the lethality of i.p. administered tubercidin.

Following the i.p. administration of tubercidin, ascitic fluid accumulated in the peritoneal cavities of mice so treated, causing abdominal distension. The development of ascites was especially pronounced in mice that received single injections of tubercidin at 5 and 10 mg/kg. The survival times (± S.D.) of these mice (18.4 ± 5.7 and 8.1 ± 6.1 days respectively) were longer than those of mice that received tubercidin at 15 mg/kg (3.9 days ± 0.6 days) and evidently allowed the accumulation of more ascitic fluid than in the high dosage mice. Examination of moribund, tubercidin-treated mice revealed other signs of peritonitis (peritoneal adhesions) and of pancreatitis (necrotic lesions). These pathological changes were not present, or were of minor degree in NBMPR-protected mice.

Because of the macroscopic evidence that tuber-

Table 1. Sequence/time dependence of NBMPR-P protection against the lethality of i.p. administered tubercidin in mice*

NBMPR-P (mg/kg)	Interval between doses of NBMPR-P and tubercidin (min)	Mortality by day 30	Mean survival time of mice that died (days ± S.D.)
0		8/8	3.5 ± 0.5
50	−360	7/8	6.3 ± 1.1
50	−120	7/8	7.6 ± 2.1
50	− 30	0/8	
50	0	1/8	20
50	+ 2	2/8†	29, 30
50	+ 5	8/8	7.3 ± 1.0
50	+ 10	8/8	4.8 ± 0.5

* Male B10D2F$_1$ mice received single i.p. injections of (i) tubercidin at time zero, and (ii) NBMPR-P either simultaneously with tubercidin, or at the time intervals indicated before (−) or after (+) the administration of tubercidin at 15 mg/kg. Deaths were recorded daily for the next 30 days.

† In three of the day 30 survivors, abdomens were distended by the accumulation of ascitic fluid.

Table 2. Dose dependence of NBMPR-P protection against the lethality of i.p. administered tubercidin in mice*

Treatment (mg/kg)				
NBMPR-P		Tubercidin	Mortality	Mean survival time of mice that died
i.v.	i.p.	i.p.	by day 30	(days ± S.D.)
		15	8/8	3.9 ± 0.6
3		15	8/8	6.5 ± 0.8
6		15	7/8	6.9 ± 1.2
12		15	8/8	4.4 ± 0.9
25		15	8/8	5.1 ± 1.1
50		15	8/8	4.6 ± 0.7
100		15	8/8	5.1 ± 1.2
	3	15	8/8	5.8 ± 1.2
	6	15	8/8	6.0 ± 1.2
	12	15	7/8	5.6 ± 1.0
	25	15	7/8	9.9 ± 7.5
	50	15	3/8	14.0 ± 7.2
	100	15	0/8	

* Male B10D2F$_1$ mice received a single i.p. or i.v. injection of NBMPR-P at the doses indicated 30 min prior to the i.p. injection of tubercidin at 15 mg/kg. Deaths were recorded daily for the next 30 days.

cidin caused pancreatic injury, the glucose tolerance of tubercidin-treated mice was investigated in the following manner. Glucose (2 g/kg) was administered p.o. (in water, 0.1 g/ml), and the urine output during the next 2 hr was assayed for its glucose content. While no glucose was detected in the urine of saline-treated control mice, nor in that of mice treated i.p. with both NBMPR-P and tubercidin, glucose was found in the urine of mice treated with i.p. tubercidin alone at 10 or 15 mg/kg. However, glucosuria in response to glucose loading was not a consistent response at long intervals after tubercidin administration.

A response to the i.p. administration of tubercidin to mice at 10 or 15 mg/kg (lethal doses, Fig. 6) was an increase (less than 2-fold) in serum activities of amylase and GPT during the first 3 post-treatment days (data not shown). As seen in Fig. 2, these changes were minor relative to those resulting from the i.v. administration of tubercidin at 30 or 45 mg/kg. Increases in serum amylase and GPT activities did not occur when a protecting dose of NBMPR-P was co-administered with tubercidin, the latter at i.p. doses of 10 or 15 mg/kg.

To test the idea that i.p. administered tubercidin caused pancreatic damage with associated release of pancreatic enzymes into the peritoneal cavity, the influence of tubercidin administration on the amylase activity in peritoneal fluids and rinsings was investigated. Intraperitoneal injections of tubercidin caused marked increases in the amylase activity of peritoneal fluids, an effect that was strongly dose dependent. The data of Fig. 7 demonstrate a relationship between (a) enhancement of amylase activity in peritoneal fluids, and (b) mortality in mice resulting from the administration of tubercidin with and without NBMPR-P.

Treatment of mice with NBMPR-P alone at 100 mg/kg, whether by the i.p. or i.v. route, did not significantly change the amylase activity in peritoneal rinsings. When NBMPR-P was administered 30 min before tubercidin injection, reduction in amylase release into the peritoneal cavity was observed. The relationship between mortality in tubercidin-treated mice and peritoneal amylase activity was not obviously changed by NBMPR-P pretreatment (Fig. 7). Experiments of this sort would not be expected

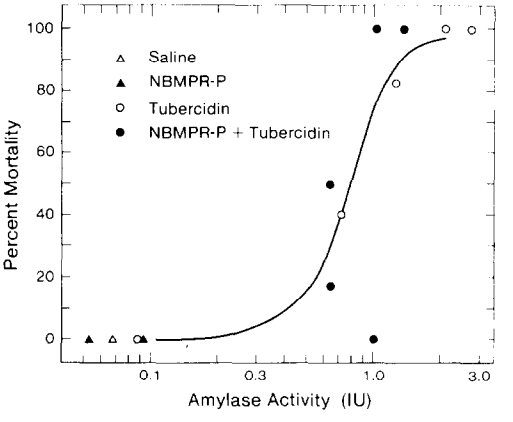


Fig. 7. Relationship between peritoneal amylase activity and lethality of i.p. administered tubercidin. Female B10D2F$_1$ mice (in groups of ten) received an i.v. or i.p. injection of NBMPR-P (doses varied from 6 to 100 mg/kg) 30 min prior to the i.p. injection of tubercidin (1–15 mg/kg); in control groups, saline was administered in place of one agent or the other. Deaths were recorded daily for the next 30 days. Four mice of each group were killed 3 days after injection of the drugs; in these animals, ascitic fluid was withdrawn, its volume was measured, and the peritoneal cavity was rinsed with 2 ml saline. Amylase activity was measured in the pooled peritoneal fluid and rinsings of each group and was expressed as peritoneal enzyme activity [International Units (I.U.)] per mouse. Drug doses (mg/kg), given i.p. except as noted, were as follows: NBMPR-P (▲), 100, 100 i.v.; tubercidin (○) 1, 3, 6, 10, 15; NBMPR-P + tubercidin (●), 25 + 10, 6 + 15, 25 + 15, 100 + 15, 100 i.v. + 15.

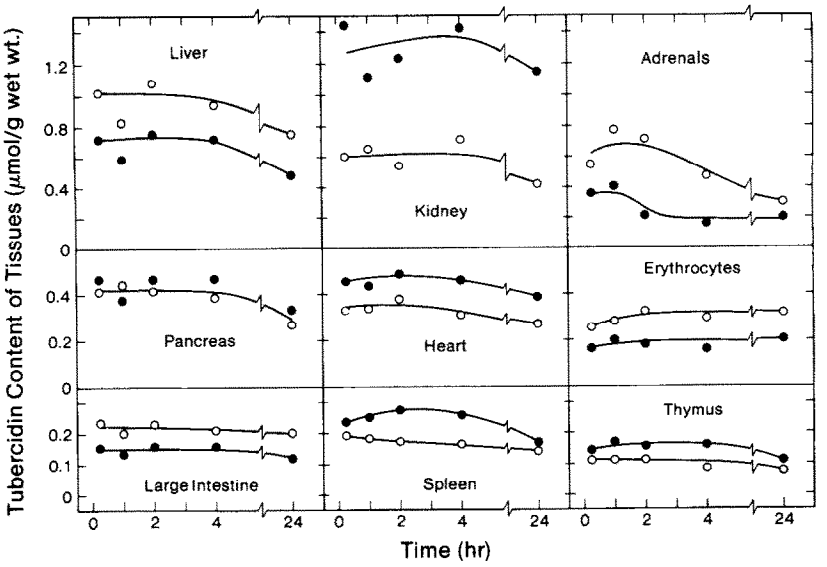


Fig. 8. Distribution of i.v. administered [^{3}H]tubercidin in tissues of the mouse. Female B10D2F$_1$ mice were injected i.p. with saline (○) or NBMPR-P (●) at 25 mg/kg 30 min prior to the i.v. injection of [G-^{3}H]tubercidin at 45 mg/kg (0.17 pmole/g body weight). At specified times after tubercidin administration, mice were killed, and the ^{3}H content of their tissues was determined. The tubercidin content of erythrocytes was derived from the difference between the ^{3}H contents of whole blood and serum. Tissue levels of ^{3}H are expressed as tubercidin equivalents. Each datum is a mean representing two to four experiments.

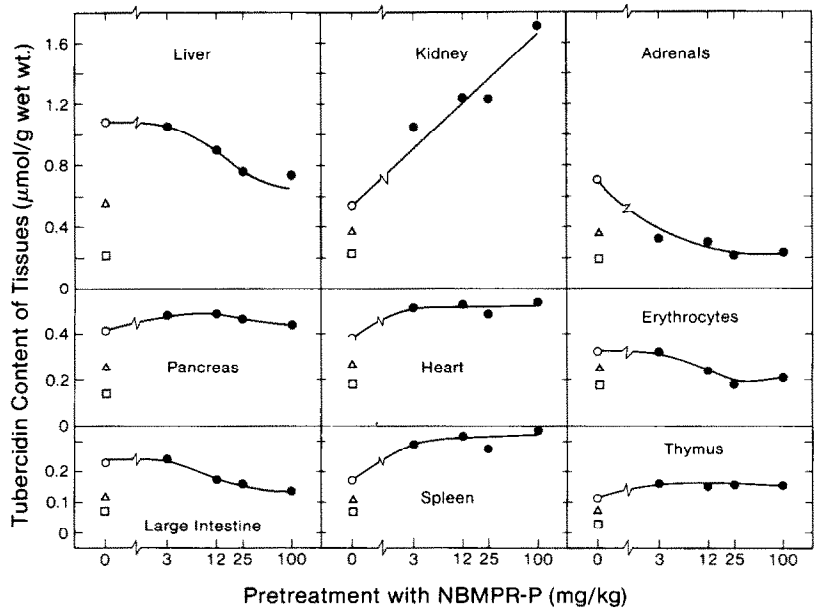


Fig. 9. Distribution of i.v. administered [^{3}H]tubercidin in tissues of the mouse: influence of tubercidin dose and NBMPR-P pretreatment. Female B10D2F$_1$ mice were injected i.p. with saline (○, △, □) or with NBMPR-P (●) at the indicated doses 30 min prior to the i.v. injection of [G-^{3}H]tubercidin at doses of 15 (□), 30 (△) or 45 (○, ●) mg/kg. Two hours after tubercidin administration, the mice were killed, and the tissue distribution of ^{3}H was determined. Tissue contents of ^{3}H are expressed as tubercidin equivalents. Each datum is a mean representing two to four experiments.

Table 3. Influence of NBMPR-P pretreatment on tissue distribution of [G-^{3}H]tubercidin in the mouse*

	Drug treatments: agents, route of administration and dose (mg/kg)						
NBMPR-P	Sal, i.p.	6, i.p.	100, i.p.	Sal, i.p.	Sal, i.p.	Sal, i.p.	25, i.p.
Tubercidin	15, i.p.	15, i.p.	15, i.p.	15, i.v.	30, i.v.	45, i.v.	45, i.v.
	Tubercidin content of tissues (nmoles/g wet weight): mean values ± S.D.						
Blood	141 ± 17	16 ± 0	16 ± 1	84 ± 13	124 ± 23	165 ± 31	93 ± 21
Serum	1.7 ± 0.3	2.6 ± 1.1	2.1 ± 0.4	2 ± 1	4 ± 2	6 ± 2	5 ± 2
Kidney	45 ± 4	368 ± 65	618 ± 79	229 ± 62	385 ± 125	541 ± 77	1233 ± 208
Liver	179 ± 8	212 ± 32	212 ± 24	212 ± 54	557 ± 135	1076 ± 207	763 ± 154
Pancreas (whole organ)	281 ± 43	261 ± 34	214 ± 2	129 ± 46	253 ± 49	412 ± 79	463 ± 47
Pancreas (isthmus†)	448 ± 57	413 ± 50	319 ± 27				
Adrenals	169 ± 17	149 ± 29	119 ± 6	204 ± 74	363 ± 87	707 ± 296	209 ± 101
Spleen	264 ± 50	168 ± 23	223 ± 21	61 ± 27	104 ± 53	172 ± 16	274 ± 67
Thymus	13 ± 8	16 ± 6	20 ± 2	24 ± 8	67 ± 29	111 ± 21	155 ± 30
Stomach	94 ± 19	91 ± 12	104 ± 84	39 ± 4	80 ± 18	105 ± 38	88 ± 12
Jejunum	164 ± 79	185 ± 45	174 ± 77	108 ± 39	234 ± 49	352 ± 71	356 ± 57
Ileum	137 ± 48	147 ± 15	123 ± 21	75 ± 14	181 ± 30	296 ± 64	333 ± 95
Large intestine	83 ± 22	77 ± 15	74 ± 6	61 ± 16	114 ± 8	230 ± 83	161 ± 32
Heart	18 ± 3	101 ± 23	123 ± 7	178 ± 21	266 ± 41	380 ± 68	488 ± 69
Lungs	111 ± 20	29 ± 5	35 ± 5	130 ± 21	242 ± 37	295 ± 95	203 ± 29
Brain	2 ± 1	1.2 ± 0.2	1.4 ± 0.2	2.6 ± 0.3	5 ± 1	6 ± 1	5 ± 1
Skeletal muscle	4 ± 1	9 ± 2	12 ± 4	36 ± 9	66 ± 21	122 ± 16	94 ± 11
Mesentery	807 ± 378	491 ± 122	349 ± 51	38 ± 12	86 ± 18	136 ± 80	138 ± 34
Number of experiments	4	4	3	4	4	4	4

* Female B10D2F$_1$ mice were injected i.p. with saline (Sal) or NBMPR-P 30 min prior to the injection of [^{3}H]tubercidin. These agents were administered at the doses specified above. Two hours later mice were killed for determination of the ^{3}H content of their tissues, expressed here as tubercidin equivalents.

† That narrow part of the pancreas which, in the mouse, lies near the stomach between the splenic and duodenal portions of this gland.

to yield more than a rough approximation of a relationship between pancreatic injury (as indicated by amylase release) and mortality. Thus, we have interpreted the appearance of amylase activity in peritoneal fluids to signify pancreatic injury; the apparent, rough relationship between peritoneal amylase activity and tubercidin-induced mortality in mice suggests that pancreatic injury may contribute to that mortality.

Influence of NBMPR-P on tissue distribution of i.v. administered [^{3}H]tubercidin. The experiments summarized in Fig. 8 show that the tissue distribution in the mouse of i.v. administered [^{3}H]tubercidin (at 45 mg/kg) did not change markedly between 15 min and 24 hr after administration. The highest level of ^{3}H occurred in liver, a finding consistent with the evidence presented above that hepatic toxicity is a consequence of tubercidin administration by the i.v. route. The i.p. treatment of mice with NBMPR-P at 25 mg/kg 30 min prior to i.v. injection of [^{3}H]tubercidin caused some changes in the tissue distribution of ^{3}H. An increase in tissue concentration of ^{3}H (representing tubercidin and its metabolites) was observed in kidneys, heart, spleen and thymus after NBMPR-P treatment, while a decrease was found in liver, adrenals, erythrocytes, large intestine and skeletal muscle; no significant changes were seen in the other organs assayed (Fig. 8 and Table 3).

We investigated the influence of variations in NBMPR-P dose on the tissue distribution of tubercidin, choosing as the distribution variable, the tissue content of ^{3}H measured 2 hr after the i.v. injection of [^{3}H]tubercidin at 45 mg/kg. The 2-hr interval was chosen because marked changes in the tubercidin content of tissues did not occur during the interval between 15 min and 24 hr after administration, as is apparent in Fig. 8. A dose-dependent decrease in the tissue content of tubercidin was observed in liver, erythrocytes and large intestine when the NBMPR-P doses were raised from 3 to 100 mg/kg (Fig. 9). When the NBMPR-P dose was 100 mg/kg, tissue levels of tubercidin in these organs were found to be close to those occurring after administration of [^{3}H]tubercidin at the non-lethal dose of 30 mg/kg. The tubercidin content of adrenals was decreased and that of heart, spleen and thymus was increased by prior administration of NBMPR-P at 3 mg/kg, but higher NBMPR-P doses were without further effect. The tubercidin content of kidneys increased with increasing doses of NBMPR-P. In mice treated with NBMPR-P at 100 mg/kg prior to administration of [^{3}H]tubercidin, the ^{3}H content of kidneys reached levels three times higher than in mice not treated with NBMPR-P (Fig. 9).

Influence of NBMPR-P on serum levels of i.v. administered tubercidin. It was expected that NBMPR-P, an inhibitor of nucleoside transport, would influence time-dependent changes in serum levels of tubercidin by impeding the entry of tubercidin into tissues. The experiment of Fig. 8 showed that, by 15 min after the i.v. administration of [^{3}H]tubercidin, the ^{3}H content of various mouse tissues had stabilized. This result indicated that, for definition of the time-dependent changes in tubercidin concentrations in serum, samples of serum would have to be obtained at very brief intervals after tubercidin administration. Blood sampling by decapitation was sufficiently rapid for this purpose but, to preclude uptake of tubercidin by erythrocytes during preparation of serum from blood samples, blood was collected into saline containing 100 μM HNBTGR, a potent inhibitor of nucleoside transport in erythrocytes [19], and cells were pelleted at once. Cellular uptake of tubercidin added to mouse blood *in vitro* in the presence of HNBTGR was less than 10% of that without HNBTGR. The experiment of Fig. 10 employed this rapid sampling method to follow tubercidin levels in serum.

After i.v. injection of [^{3}H]tubercidin at 45 mg/kg, the ^{3}H content of serum declined with a half-life of about 10 sec during the first minute after tubercidin administration (Fig. 10). Thereafter, the ^{3}H content of serum remained approximately constant during the next 24 hr. Treatment of mice with NBMPR-P (25 mg/kg) 30 min prior to the injection of [^{3}H]tubercidin markedly reduced the rate at which [^{3}H]tubercidin levels in serum diminished. In NBMPR-P-treated mice, the ^{3}H content of serum declined during the first minute after tubercidin administration with a half-life of about 26 sec; thereafter, the decline was slower, and serum levels of ^{3}H approached those in untreated mice after 5 min. One

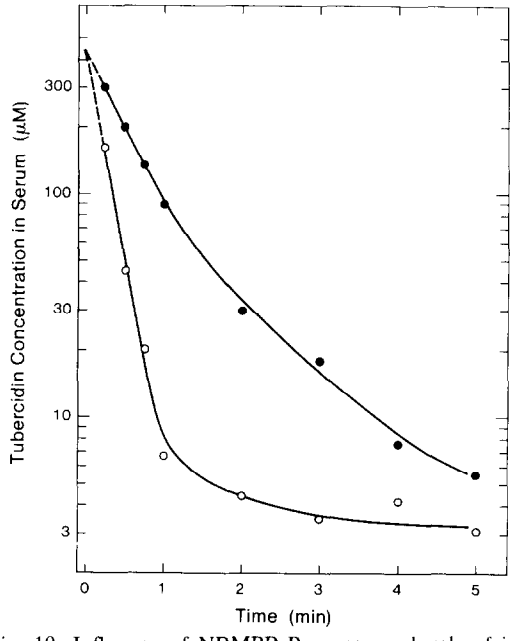


Fig. 10. Influence of NBMPR-P on serum levels of i.v. administered [G-^{3}H]tubercidin. Female B10D2F$_1$ mice were injected i.p. with saline (○) or NBMPR-P (●) at 25 mg/kg 30 min prior to the i.v. injection of [G-^{3}H]tubercidin at 45 mg/kg. At the indicated times after tubercidin administration, mice were decapitated and a blood sample from each was collected in a known volume of saline containing 100 μM HNBTGR and [^{14}C]inulin. After immediate pelleting of cells, ^{3}H and ^{14}C activities in the diluted serum samples were determined by a dual label combustion method. Each datum represents a single assay. Since inulin does not enter cells, the volume of serum in the samples collected was derived from the dilution of the [^{14}C]inulin which occurred during the sampling procedure.

minute after injection, the tubercidin concentration of serum in NBMPR-P-treated mice was more than 10-fold higher than that in untreated mice. The data of Fig. 10 indicate that the extrapolated time-zero concentration of tubercidin in serum (about 440 μM) was independent of NBMPR-P treatment.

Influence of NBMPR-P on tissue distribution of i.p. administered [^{3}H]tubercidin. Pilot experiments showed that, after a single i.p. injection of [^{3}H]tubercidin at 15 mg/kg, no significant changes in the tissue distribution of tubercidin occurred during the interval between 15 min and 24 hr after injection. The present study evaluated the influence of NBMPR-P on the tissue distribution of tubercidin measured 2 hr after the i.p. administration of the toxic nucleoside. Thirty minutes prior to the i.p. injection of [^{3}H]tubercidin at 15 mg/kg, mice received NBMPR-P i.p. at (i) 6 mg/kg [no protection against tubercidin lethality (Table 2)], or (ii) 100 mg/kg (protection against tubercidin lethality).

Table 3 compares tissue levels of tubercidin-derived ^{3}H measured 2 hr after administration of [^{3}H]tubercidin by (i) the i.p. route at 15 mg/kg (a lethal dose), and (ii) the i.v. route at the nonlethal doses of 15 and 30 mg/kg (Fig. 1). The ^{3}H content of several tissues from the peritoneal cavity (mesentery, stomach, intestine, pancreas and spleen) was higher in i.p. injected animals than in those which received the drug by the i.v. route; direct contact of tissue surfaces with the i.p. administered drug probably contributed to ^{3}H uptake. The ^{3}H content of erythrocytes was also higher when the labeled nucleoside was given by i.p. injection, whereas that route of administration gave rise to a lower content of tubercidin-derived ^{3}H in kidneys, thymus, heart and skeletal muscle than did i.v. administration.

When mice received i.p. NBMPR-P prior to i.p. administration of [^{3}H]tubercidin, ^{3}H levels in erythrocytes, adrenals, lungs, pancreas and mesentery were reduced. Such reduction in the content of tubercidin and metabolites in erythrocytes, adrenals and lungs was evidently not related to NBMPR-P protection against tubercidin lethality because similar and higher tubercidin concentrations were found in those tissues in mice that received only i.v. [^{3}H]tubercidin at 30 mg/kg, a tolerated dose (Fig. 1 and Table 3).

The inhibition by NBMPR-P of tubercidin uptake by pancreas and mesentery is consistent with the protective effects of NBMPR-P against the pancreatic injury and peritonitis which result from the i.p. administration of tubercidin. However, the quantitative aspects of that reduction in tubercidin uptake by pancreas (24%) and mesentery (57%) are difficult to reconcile with the fact that a 10-fold reduction in dose was required to avoid lethality from the i.p. administration of tubercidin alone (Fig. 6). With pancreas, these apparent inconsistencies may derive from the inability of our methods to relate drug content to injury in particular portions of that tissue. This possibility was suggested by the observation that the isotope concentration in that tissue was not uniform, being highest in the isthmus of the pancreas (Table 3) where tubercidin-induced damage was observed macroscopically.

It is apparent in Table 3 that NBMPR-P treatment caused large increases in the kidney content of tubercidin-derived ^{3}H when [^{3}H]tubercidin was administered i.p. or by the i.v. route.

DISCUSSION

The manifestations of tubercidin toxicity in B10D2F$_1$ mice that we report here are similar to those previously observed [20–25]. Liver is a primary site of tubercidin injury in mice after i.v. administration of this agent, as indicated by jaundice, large increases in GPT activity and bilirubin levels in serum and, as well, by histological evidence of liver injury, all found several days after a single, potentially lethal dose of tubercidin. These indicators of liver damage were shifted toward normal in NBMPR-P-treated mice that had received the same dose of tubercidin. Prevention of liver injury would appear to be an important aspect of NBMPR-P protection against potentially lethal doses of tubercidin. Injury of the pancreas seems not to contribute importantly to mortality from i.v. tubercidin because NBMPR-P treatment decreased mortality without preventing pancreatic damage which was apparent histologically and in the elevation of serum amylase activity.

In general, NBMPR-P effects on the tissue distribution of tubercidin were consistent with NBMPR-P protective effects against tubercidin toxicity and lethality. Tissue distribution of [^{3}H]tubercidin was monitored by measuring only the tissue content of ^{3}H; metabolites of tubercidin were not determined. While relationships between tissue content of tubercidin and effect might have been more clearly perceived if tissue content had been defined in terms of tubercidin metabolites, we have, nevertheless, shown that NBMPR-P-induced reduction in the liver content of tubercidin-derived ^{3}H correlated with reduction both in mortality and biochemical signs of liver toxicity from i.v. administered tubercidin. In experiments to be reported elsewhere, we have shown that in isolated, perfused livers of mice, a major component of cellular uptake of tubercidin is blocked by NBMPR (N. Kolassa, P. M. Roth and A. R. P. Paterson, unpublished results).

The following evidence argues that injury to the peritoneal epithelium and underlying tissues, particularly to pancreas, contributed importantly to mortality in mice treated with tubercidin by i.p. injection. First, because the LD_{50} for i.p. administered tubercidin (5 mg/kg) was about 7-fold less than that of the i.v. administered drug, the i.p. doses of tubercidin here employed were lower than i.v. doses. Hence, the apparent absence of liver damage in response to i.p. tubercidin in these experiments was probably a matter of dose. Second, mortality from i.p. administered tubercidin was reduced by NBMPR-P given by the same route, but not by i.v. doses of the protecting agent. Third, pronounced ascites and pancreatic injury occurred together with the appearance of amylase activity in peritoneal fluid. Concomitant increases in serum amylase activity were minor. Fourth, of the mouse tissues examined after i.p. administration of [^{3}H]tubercidin, the highest levels of ^{3}H were found in pancreas and mesentery. These levels were reduced by NBMPR-P. Taken together, these findings indicate

NBMPR-P. Taken together, these findings indicate that NBMPR-P inhibition of tubercidin uptake by pancreas and peritoneal epithelium is the probable basis of NBMPR-P protection against the toxicity of i.p. injected tubercidin.

Significant delay by NBMPR-P of the movement into the circulation of i.p. administered tubercidin would not be expected since paracellular permeation is the main pathway for the exchange of drugs between the peritoneal cavity and blood [26]. We have observed that NBMPR-P treatment resulted in only minor reduction in rates at which tubercidin or purine riboside left the peritoneal cavities of mice (N. Kolassa, J. H. Paran and A. R. P. Paterson, unpublished results). Thus, i.p. administered tubercidin is delivered by the circulation to organs, and cellular uptake of tubercidin in some organs is modified by NBMPR-P treatment.

The expectation that NBMPR-P, a soluble "prodrug" form of NBMPR, would impede the entry of tubercidin into tissues was met in experiments which showed that NBMPR-P treatment markedly reduced the early rate at which tubercidin was cleared from the circulating plasma in mice. However, the distribution of tubercidin into mouse tissues is a rapid process, and NBMPR-P-induced differences in the rates at which tubercidin serum levels declined were of such short duration that substantial changes in the renal excretion of tubercidin did not follow NBMPR-P treatment.

It will be noted that NBMPR-P treatment enhanced tubercidin uptake by some mouse tissues. How may the apparently contradictory effects of NBMPR-P (enhancement of tubercidin uptake in some tissues and inhibition in others) be explained? First, rates of NBMPR-P conversion to NBMPR, the actual inhibitor, may differ between tissues with the consequence that a dose of NBMPR-P may not produce uniform inhibitory effects on nucleoside transport in various tissues. Second, rates of residual, NBMPR-insensitive permeation of tubercidin may differ among tissues. Third, differences in the kinetic characteristics of nucleoside transport (in particular, V_{max}) between tissues may contribute to the disparate effects of NBMPR-P on tubercidin distribution that we report here.

Large increases in tubercidin accumulation by kidneys resulted from the i.p. administration of NBMPR together with either i.p. or i.v. tubercidin. This effect was consistent with the result that a high dose of NBMPR-P (100 mg/kg) given 30 min prior to i.v. tubercidin at 45 mg/kg was severely nephrotoxic, whereas tubercidin alone at this dose caused no apparent kidney damage.

Kidneys in mice are heavily labeled after the i.v. injection of [G-^{3}H]NBMPR-P (S. M. Jarvis, L. L. Lam and A. R. P. Paterson, unpublished results), an observation consistent with the high phosphomonoesterase activity in mouse kidney [27]. Thus, the presence of NBMPR was probably responsible for the large, NBMPR-P-induced increase in tubercidin accumulation in this organ. Permeants enter kidney tubular cells either from the blood side or, after glomerular filtration, from the lumen side of the tubule. With the assumption that nucleoside permeation is differently affected by NBMPR at the luminal and contraluminal membranes of the tubular cells, the tubercidin content of the latter could be increased by NBMPR-P in either of the following ways: (i) if the permeation of tubercidin across the luminal membrane were preferentially inhibited, the passage of tubercidin from tubular cells into the tubule lumen would be blocked with the effect that cellular tubercidin would be retained, or (ii) if the permeation of tubercidin across the contraluminal membrane were preferentially inhibited, the passage of tubercidin from tubule cells into blood would be blocked. It is assumed that in (i) a preferential secretion of tubercidin would occur across the tubular epithelium from blood to lumen, and that in (ii) a preferential absorptive flow from lumen to blood would occur. Either of these circumstances would lead to NBMPR-P-induced accumulation of tubercidin in tubular cells. Differential inhibition of nucleoside permeation at the luminal and contraluminal membranes of jejunal epithelia in guinea pigs has been observed in the presence of nitrobenzylthioguanosine or other nucleoside transport inhibitors [28, 29]. The inhibitors of nucleoside transport may be useful in understanding the handling of nucleosides by the kidney and may enable some manipulation of nucleoside disposition *in vivo*.

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