Pharmacological Studies of the Antitumor Agent 6-Methylthiopurine Ribonucleoside

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SUMMARY

The distribution of 6-methylthiopurine ribonucleoside (MMPR) and its metabolites was studied in BDF/1 mice after a single i.p. injection of MMPR-35S. One hr following the administration of MMPR, almost one-half of the administered radioactivity could be accounted for in the liver and red blood cells, with 7% in the intestinal mucosa and very small amounts in the kidney, spleen, and plasma. Almost all of the radioactivity in these tissues could be accounted for as MMPR and its 5'-monophosphate, 6-methylthiopurine ribonucleotide (MMPR-P). The biological half-time of the total radioactivity in the different tissues varied from 10 to 18 hr. The disappearance rate for the unchanged drug, MMPR, or the metabolite, MMPR-P, was parallel to that of the total radioactivity. In red blood cells, all of the radioactivity was in the form of MMPR-P. There was progressively less MMPR-P and more MMPR in the liver, kidney, and intestinal mucosa.

Adenosine kinase and purine nucleotide phosphohydrolase (MMPR-P to MMPR) activities were determined in a variety of human and mouse tissues. There was some correlation between the uptake of MMPR and the ratios of MMPR-P to MMPR and adenosine kinase to phosphohydrolase activity in mouse tissues. With one exception, the enzyme activities for the tissues studied were very similar for the mouse and man. The exception is the extremely high phosphohydrolase activity in the mouse kidney.

The urinary excretion of MMPR and its metabolites following MMPR-35S administration was studied in mouse and man. Approximately 40% of the total radioactivity was excreted in 24 hr in the mouse, compared to 37% in man. There were 6 products in the urine of mouse, but only three were found in man. The major identified products were MMPR and inorganic sulfate, and little or no MMPR-P could be detected in the urine of either species. As early as 2 hr following MMPR-35S injection, an appreciable amount of inorganic sulfate could be detected in the urine of both mouse and man, and the sulfate excretion increased steadily with time. However, the rate of sulfate excretion was faster in man, and, at 4 hr, 80% of the excreted radioactivity was inorganic sulfate. This level was maintained for at least 5 days.

The pharmacological studies of MMPR in the mouse and in man are compared and discussed.

INTRODUCTION

MMPR2 is an adenosine analog with substantial antitumor activity in experimental systems (1, 12). It is not cross-resistant with 6-mercaptopurine; i.e., it maintains its activity in L1210 mouse leukemia and human cancer cells in culture, which are resistant to 6-mercaptopurine (2, 4). The biochemical basis for this lack of cross-resistance has been established (1, 4, 14). Resistance to the thiopurines often results from deletion of the enzyme hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8), which is essential for the conversion of the base to the active intermediate, the ribonucleotide. However, the phosphorylation of MMPR, an adenosine analog, is mediated through adenosine kinase (EC 2.7.1.10) (2, 5), which persists in cell lines resistant to 6-mercaptopurine. In addition, MMPR in combination with 6-mercaptopurine is synergistic both in the Ehrlich ascites carcinoma (18) and in the L1210 mouse leukemia (17). These important experimental observations have been extended to the clinic. It has been found that MMPR is not effective in treatment of acute leukemia refractory to 6-mercaptopurine (11). However, MMPR in combination with 6-mercaptopurine is effective in inducing remission in adults with acute myelogenous leukemia (3).

Because of the above biological and biochemical observations in experimental systems, pharmacological studies of MMPR in the mouse have been undertaken. In an earlier study in man, the major urinary metabolite excreted was reported to be MMPR-P (9). The excretion and metabolism of MMPR has been further studied in man, and the current report is concerned with the comparative biochemical pharmacology of MMPR in the mouse and in man.

MATERIALS AND METHODS

MMPR and MMPR-35S were supplied by the National Cancer Institute, Bethesda, Md. The other chemicals are commercially available. Cellulose MN 300 thin-layer chromatography plates were purchased from Mann Research Laboratories, Inc., New York, N. Y. Male BDF/1 mice, 1.5 to 2 months old, were used (Texas Inbred Mouse Company, Houston, Texas). The mice were given i.p. injections of 60 mg/kg of MMPR with 3.2 μCi/mg of 35S-labeled MMPR. The radiochemical purity of MMPR was over 97%, as determined by Dr. John A. Montgomery, Southern Research Institute.

1 This investigation was supported in part by Contract PH 43-67-1156 and Grant CA 05831 from the National Cancer Institute, Bethesda, Md.

Received June 15, 1970; accepted August 6, 1970.
The administration of 5'-nucleotidase of Crotalus adamanteus venom (7), the assay of adenosine kinase, with MMPR-35S as a substrate, has been reported elsewhere (2, 5, 7).

For assay of phosphohydrolase activity, the incubation mixture consisted of 20 μmoles of glycine buffer, pH 9.5, 0.075 μmole of MMPR-P-35S, and an appropriate amount of enzyme in a total volume of 0.4 ml. The reaction was carried out at 37°C for 5 min. With thin-layer chromatographic, radio-scanning, and counting techniques (7), the relative amounts of MMPR-P and MMPR were measured quantitatively. The optimum pH for adenosine kinase is 5.4 with succinate buffer (7), and there is essentially no adenosine kinase activity at pH 9.5. In addition, the adenosine kinase assay requires ATP.

Thus, at pH 9.5 and in the absence of ATP, the phosphohydrolase can be assayed without the interference of adenosine kinase activity. Since no other products have been found in in vitro reactions, this assay system is relatively simple. This method will be presented in detail elsewhere.

Protein concentration was determined by the method of Lowry et al. (10), with bovine serum albumin as a standard.

Chart 1. Drug distribution in mouse tissues after administration of MMPR-35S i.p. A, plasma and red blood cells; B, liver; C, kidney; and D, intestinal mucosa. The drug was given at time zero and the assays were made at the intervals indicated.
Table 1

Distribution and metabolism studies of MMPR in mouse and man

One hr following i.p. administration of MMPR-35S to mice, the tissues were analyzed for the total radioactivity and metabolites.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% of administered radioactivity/g of tissue or ml of fluid</th>
<th>Ratio of MMPR-P to MMPR</th>
<th>Half-time (hr)</th>
<th>In vitro enzyme activity of</th>
<th>Ratio (A:B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenosine kinase (A)</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td>Phosphohydrolase (B)</td>
<td></td>
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<tr>
<td>RBC</td>
<td>26.0</td>
<td>infinite</td>
<td>18</td>
<td>316.0</td>
<td>0.9</td>
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<tr>
<td>Liver</td>
<td>25.0</td>
<td>4.1</td>
<td>10</td>
<td>85.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>7.5</td>
<td>1.5:1</td>
<td>10</td>
<td>7.6</td>
<td>38.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.1</td>
<td>2.1</td>
<td>14</td>
<td>29.2</td>
<td>125.0</td>
</tr>
<tr>
<td>Spleen</td>
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<td>&lt;1.0</td>
<td></td>
<td>7.6</td>
<td>10.4</td>
</tr>
<tr>
<td>Plasma</td>
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<td>&lt;1.0</td>
<td></td>
<td>0.0</td>
<td>17.4</td>
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<tr>
<td>Human</td>
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<td></td>
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<td>infinite</td>
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<td>139.0</td>
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<tr>
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<td>16.0</td>
<td>7.0</td>
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<tr>
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<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td>0.0</td>
<td>10.8</td>
</tr>
</tbody>
</table>

a Specific activity for tissues, moles of product/mg of protein/min at 37°; specific activity for RBC and plasma, moles of product/ml/min at 37°.
b Taken from Ref. 9.

RESULTS

The drug and radioactivity distributions in the plasma and red blood cells of BDF/1 mice are presented in Chart 1. The slopes of the disappearance of radioactivity from the red blood cells and plasma are exponential and comparable. The half-time for drug disappearance of both tissues was 18 hr. The concentration of radioactivity within the red blood cells at all times was approximately 30 times that in the plasma. All of the radioactivity in the red blood cells could be accounted for as 6-methylthiopurine S'-monophosphate. The very low concentration in the plasma samples precluded precise quantitative determination of metabolites. Approximately 30% of the radioactivity could be accounted for as MMPR. Another identified peak in the plasma was sulfate, and sulfate was present in the urine (see below). There was no sulfate in the red blood cells, and less than 5% of the radioactivity in other tissues was sulfate.

Similar analyses were made of the kidney, liver, and intestinal mucosa (Chart 1). In the liver, almost all of the radioactivity could be accounted for as MMPR-P and MMPR. Up through 48 hr, 80% of the radioactivity was composed of the nucleotide (MMPR-P), and the half-time in this tissue was 10 hr. In the kidney, almost all of the radioactivity could be accounted for as the nucleotide and nucleoside with approximately 2/3 of the radioactivity in the form of the nucleotide. The half-time in this tissue was 14 hr. In the intestine, 60% of the radioactivity was in the form of MMPR-P, and again almost all of the radioactivity could be accounted for by the unchanged drug and nucleotide. The half-time in the intestine was 10 hr.

These data are summarized in Table 1. One hr following the administration of MMPR, the highest concentration of radioactivity in the plasma and red blood cells of BDF/1 mice are presented in Chart 1. The slopes of the disappearance of radioactivity from the red blood cells and plasma are exponential and comparable. The half-time for drug disappearance of both tissues was 18 hr. The concentration of radioactivity within the red blood cells at all times was approximately 30 times that in the plasma. All of the radioactivity in the red blood cells could be accounted for as 6-methylthiopurine S'-monophosphate. The very low concentration in the plasma samples precluded precise quantitative determination of metabolites. Approximately 30% of the radioactivity could be accounted for as MMPR. Another identified peak in the plasma was sulfate, and sulfate was present in the urine (see below). There was no sulfate in the red blood cells, and less than 5% of the radioactivity in other tissues was sulfate.

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high phosphohydrolase activity in mouse kidney. The specific activity in the mouse kidney is 125 numoles of product/mg of protein/min, compared to only 6.5 in the human kidney.

The urinary excretion of MMPR as total radioactivity in the mouse and man following MMPR-35S administration is presented in Chart 2. As presented in Fig. 1, the radioautogram showed that at least 6 compounds were found in the mouse urine. The lower portion of the first spot (near solvent front) was identified as MMPR. The urine was incubated with adenosine kinase, and the radioactivity of this peak was shifted from the RF of MMPR to that of MMPR-P. The last spot (near the origin) on the radioautogram was identified as sulfate. The radio-scanning data showed that the eluted sulfate peak is completely removed after barium chloride is added in the presence or absence of 0.6 N HCl.

Studies of urinary metabolites were carried out in 3 patients who each received a single i.v. injection of MMPR-35S. Three compounds were found in the urine. The identified ones are MMPR and sulfate, and they comprised approximately 90% of the urinary radioactivity. No MMPR-P could be detected, i.e., no change was observed in RF values of any radioactive spots following incubation of urine samples with 5'-nucleotidase (Fig. 2). The sulfate could be detected as early as 2 hr after the injection, but the major radioactivity at that time was MMPR (Fig. 2). However, 24 hr postinjection, neither MMPR nor MMPR-P could be found, and there were only a major sulfate peak (near origin) and a small unidentified peak (Fig. 2). A comparison of the urinary excretion of the inorganic sulfate was made in mouse and man and is presented in Chart 3.

DISCUSSION

The rapid uptake and steep concentration gradient of MMPR in the red blood cells observed in the above experiments were similar to observations made in vivo in man (9) and in cells in vitro (8). However, the half-time of radioactivity in the red blood cells and plasma is much longer (4 to 6 days) in man (9) than in the mouse. Thus, the drug and its major metabolite MMPR-P disappear much more rapidly from the mouse tissues than from human tissues. The basis for the concentration gradient within the cells has been explained by diffusion of the relatively nonpolar nucleoside across the cell membrane and rapid conversion within the cell to the nucleotide (MMPR-P). The nucleotide is more polar and less lipid soluble and thus does not readily egress from the cell. It is proposed that the establishment of an intracellular concentration gradient and the relative amounts of MMPR-P and MMPR within a tissue are dependent upon the relative activities of adenosine kinase and the phosphohydrolase, as illustrated in Chart 4 and Table 1. In terms of uptake in mouse tissues, such a correlation appears to exist. Thus, the highest ratios for adenosine kinase to phosphohydrolase were obtained in the liver and red blood cells, and these organs, in fact, contained at 1 hr a greater percentage of the radioactivity than any other organs studied. Similarly, within these tissues, the ratio of MMPR-P to MMPR is much higher. In the red blood cells of both mouse and man, the extremely high adenosine kinase-to-phosphohydrolase ratio presumably explains the fact that essentially all of the radioactivity within these tissues is accounted for by the nucleotide, MMPR-P.

The half-time for radioactivity (Table 1) of all of the tissues in the mouse varied from 10 to 18 hr, being somewhat longer in the red blood cells than in most other tissues. Thus, there was no correlation between the half-time of the drug within a given tissue and the enzyme ratios or the ratio of nucleotide to nucleoside. While cellular turnover in the intestinal mucosa could account for its shorter half-time, this explanation would not apply to the liver or red blood cells.

Since MMPR-P is a relatively more polar compound ordinarily retained in cells, it is not likely to be excreted as a major metabolite of MMPR. Furthermore, there was little or no MMPR-P found in the mouse urine. The above studies have indicated that, in contrast to the previous report (9), sulfate, not MMPR-P, is the major excretory product in man. The excretion of inorganic sulfate-35S from humans has been reported after administering 6-methylmercaptopurine-35S (6). The major differences in the pharmacology of MMPR in the mouse and in man are shown in Table 2. The disappearance of radioactivity from the plasma and of MMPR-P from the red

Chart 3. Comparison of the urinary excretion of the inorganic sulfate in man and mouse after MMPR-35S. ©, Patient L. J. received 0.6 mg/kg of MMPR; •, Patient F. M. received 3.0 mg/kg of MMPR; □, ■, mice received 60 mg/kg of MMPR. (Each point represents 5 mice in a group). The inorganic sulfate was precipitated by adding barium chloride in the absence or presence of 0.6 N HCl.
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This observed difference could be due to individual variations in metabolism. The excretion of radioactivity in the mouse, in which 40% was excreted in the 1st day (Chart 2), is similar to the results found for the human urine, with 37% excreted in the 1st day (Table 2). It remains possible that the metabolites produced in the mouse could, in part, be responsible for the antitumor activity. Following incubation of mouse urine samples with xanthine oxidase, nucleoside phosphorylase (16), or glucosylase (a mixture of β-glucuronidase and sulfatase), no change was observed in the intensity of Rf values of the radioactivity of unknown peaks. These preliminary studies seem to exclude the possibility of the presence of metabolites such as 6-mercaptopurine, 6-mercaptopurine ribonucleoside, or their conjugated form of sulfate or glucuronide. In addition, the Rf values of these metabolites do not correspond with those of 6-thiouric acid, 2,8-dihydroxy-6-mercaptopurine, or 8-hydroxy-6-methylthiopurine. The possibility of the hydroxylation of methylthiopurine ribonucleoside at position 2 or 8 or 2 and 8 has not been eliminated. It is also possible that one of the unknown metabolites could be a disulfide, which is formed from the demethylation of the methylmercaptopurine linkage and then the oxidation of the mercaptoguanine.

The major stimulus to the above studies was the fact that MPPR was an important antitumor agent in experimental systems (1, 10) and, in combination with 6-mercaptopurine, was effective in the treatment of adults with acute leukemia (3). MPPR alone is effective in the treatment of L1210 leukemia and Ehrlich ascites carcinoma, whether sensitive or resistant to 6-mercaptopurine (2, 3). However, MPPR alone proved ineffective in the treatment of acute leukemia in man regardless of whether the patients had previously received 6-mercaptopurine (10). Because of the major interspecies difference in pharmacology, different dose schedules of MPPR administration, alone and in combination with 6-mercaptopurine, are under study in the mouse and in patients with leukemia in an effort to determine whether this important activity demonstrated against experimental leukemias can be reproduced in man.

ACKNOWLEDGMENTS

We extend our appreciation to Miss Beverly Thetford for her capable technical assistance; to Dr. John A. Montgomery of the Southern Research Institute and to the Cancer Chemotherapy National Service Center for supplying both 35S-labeled and unlabeled MPPR and MPPR-P; and to Dr. Roland Robins of the International Chemical and Nuclear Corporation, Irvine, Calif., for the gift of 8-hydroxy-6-methylthiopurine. We thank Dr. G. A. LePage for his encouragement and discussion and for synthesizing the MPPR-35S used in the studies of the urinary excretion of MPPR in man.

REFERENCES


Fig. 1. Radioautogram of a thin-layer chromatogram of mouse urine after administration of MMPR-$^{35}$S i.p. Solvent system, 44% propionic acid and 93.8% n-butyl alcohol (1:1).

Fig. 2. A radioautogram of paper chromatograms shows the results of incubating human urinary samples with adenosine kinase and 5'-nucleotidase compared to controls. Solvent system, 44% propionic acid and 93.8% n-butyl alcohol (1:1). A, 2-hr urine sample incubated without and with adenosine kinase, and the $R_F$ value of the radioactivity is changed from MMPR to that of MMPR-P after the incubation. B, control of adenosine kinase: the $R_F$ value of $MP\rightarrow S$ is changed to that of $MP\rightarrow S$ after incubation. C, no change is observed in $R_F$ values of the radioactivities of 2-hr urine sample incubating with adenosine kinase. The 2 peaks are a major inorganic sulfate (near origin) and a minor unknown (near solvent front). No change is observed in $R_F$ values of the radioactivities in urine samples of 2-hr (D) and 24-hr (F) incubated with 5'-nucleotidase. E, control of 5'-nucleotidase. The $R_F$ value of MMPR-P-$^{35}$S is changed to that of MMPR-$^{35}$S after incubating with the enzyme.
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