Effect of Methylthioinosine on Nucleotide Concentrations in L5178Y Cells

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SUMMARY

Methylthioinosine (MMPR) inhibited proliferation of cultured cells of mouse lymphoma L5178Y when present in the culture medium at concentrations of 0.2 μM or greater. The cells accumulated MMPR in the form of the 5'-monophosphate, reaching steady-state concentrations that were 500 to 600 times that of extracellular MMPR. Upon transfer to MMPR-free medium, intracellular MMPR 5'-monophosphate concentrations declined, indicating turnover of this compound.

Incubation of L5178Y cells in medium containing 0.5 μM MMPR reduced intracellular concentrations of adenosine triphosphate, guanosine triphosphate, and adenosine diphosphate by about 70% and proliferation rates by about 30%. Upon transfer of such cells to MMPR-free medium, nucleotide levels and proliferation rates returned to control values. L5178Y cells incubated in medium containing 0.1 μM MMPR proliferated at control rates even though their purine ribonucleotide levels were reduced by as much as 40%.

The reduction in cellular pools of purine nucleotides during culture in 0.1 μM MMPR was accompanied by an increase in the pyrimidine ribonucleotide concentrations. Cellular concentrations of uridine triphosphate and cytidine triphosphate increased almost threefold during the first 2 hr of exposure to 0.1 μM MMPR, then fell during the next 2 hr to a steady-state level of about 180% of control values.

INTRODUCTION

MMMPR which inhibits growth of certain transplantable tumors (2, 21) and of tumor cells in culture (2, 18, 22), is converted in sensitive cells to the 5'-monophosphate ester, MMPR-P, in a kinase-mediated reaction (2, 4, 5, 10, 19). Synthesis of MMPR-P by way of the aglycone is unlikely because MMPR is cleaved phosphorolytically very slowly (13, 19) and the free base is not a substrate for adenine or hypoxanthine-guanine phosphoribosyltransferases (14, 15). MMPR-P is a potent inhibitor of PRPP amidotransferase (9, 11), which catalyzes the 1st step of the de novo pathway of purine nucleotide synthesis. This inhibition is probably the basis of the antiproliferative effects of MMPR toward cultured cells of mouse adenocarcinoma 755 and mouse lymphoma L5178Y because proliferation rates are restored to near-control values by addition of hypoxanthine to MMPR-inhibited cultures of these cells (1) (S. C. Kim and A. R. P. Paterson, unpublished results).

The studies reported here examine MMPR-induced changes in sizes of nucleotide pools in cultured L5178Y cells and attempt to correlate such effects with the antiproliferative activity of this compound.

MATERIALS AND METHODS

Methyl-14C-labeled MMPR and MMPR-P were prepared by methods given previously (4). The MMPR-14C was shown to be at least 99% radiochemically pure by paper chromatography in butanol:acetic acid:water (60:15:25). Unlabeled MMPR was provided by Drug Research and Development, National Cancer Institute, Bethesda, Md.

Cells of mouse lymphoma L5178Y were cultured in Fischer's medium (8) supplemented with 10% horse serum, streptomycin (100 μg/ml), and penicillin (100 units/ml), as described previously (22). Cell numbers and mean cell volumes were measured with a Model F Coulter Counter calibrated for cell volume determinations with pollen grains of known dimensions. Proliferation rates were determined as the number of cell population doublings in the cultures after a specified interval and, for MMPR-treated cultures, are expressed as percentages of rates in control cultures (in which medium did not contain MMPR).

The metabolism of MMPR and its effects on sizes of cellular nucleotide pools were studied as follows. MMPR (10 mM in 0.154 M NaCl) was added to 120-ml cultures containing 2.4 × 10^7 L5178Y cells, and after measured time intervals at 37° individual cultures were cooled in ice water and centrifuged (4°, 2000 × g, 3 min) to collect cells. Cells were resuspended in 100 ml of cold medium and, after recovery by centrifuging (4°, 2000 × g, 3 min), cell pellets were mixed with perchloric acid at 4° (final volume and concentration, 0.2 ml and 0.4 M, respectively). After 15 min, the extraction mixtures were centrifuged and each acid-insoluble fraction was washed with 0.1 ml of 0.4 M perchloric acid.
perchloric acid. Washings and 1st extracts were combined and pH values were adjusted to 5 to 6 with potassium hydroxide. After 2 to 3 hr at 4°C, the extracts were centrifuged to remove precipitated potassium perchlorate and then concentrated at 4°C by evaporation under a stream of CO₂-enriched air. Final volumes were determined gravimetrically.

Nucleotide concentrations in the perchloric acid extracts were determined by high-pressure anion-exchange chromatography using a Varian Aerograph LCS-1000 and the elution system of Brown (3). Retention times and response factors for individual nucleotides were determined empirically using standards of known concentration. Peak areas in the elution profiles were estimated by multiplying the height of the peak by the width at half-height. Elution profiles from extracts of L5178Y cells had well-defined peaks representing GTP, ATP, UTP, CTP, ADP, and NAD; however, ATP and GTP were not well separated and the ATP peak partially obscured the smaller GTP peak; similarly, the CTP peak was partly obscured by the much larger UTP peak.

In the measurement of MMPR uptake, the procedure followed differed only from the above in that MMPR-¹⁴C and 50-ml cultures containing 2 × 10⁷ cells were used. Incubation periods were terminated by addition of an equal volume of cold medium and immediate centrifugation. Perchloric acid extracts of the cell pellets were prepared as described above. To determine cellular concentrations of MMPR-P-¹⁴C, measured portions of such extracts were chromatographed on paper using authentic MMPR and MMPR-P as carriers; after development in the butanol:acetic acid:water system, carrier spots were located by their fluorescence under UV and the radioactivity associated with each was measured by counting chromatogram sections directly in a liquid scintillation system.

RESULTS

As Chart 1 indicates, MMPR inhibited the proliferation of cultured L5178Y cells; when the culture medium contained 0.5 µM MMPR, cell numbers increased by about 50% during the 1st 24 hr of culture and decreased thereafter. Initial proliferation rates were somewhat higher when MMPR concentrations were between 0.2 and 0.5 µM, but proliferation rates declined after 24 hr of MMPR exposure. MMPR concentrations of 0.1 µM or less had no appreciable effect on proliferation of the lymphoma cells.

Chart 2 illustrates the time course of MMPR-¹⁴C uptake by L5178Y cells; it is seen that, after 5 to 10 min of incubation, rates of ¹⁴C uptake declined until steady-state concentrations were reached. When perchloric acid extracts of cells collected in the steady-state condition were examined by paper chromatography, their ¹⁴C content was found almost entirely (90% or more) in the form of MMPR-P.

Chart 2 shows that the steady-state concentrations of MMPR (and metabolites) achieved in the tumor cells were a function of the concentration of MMPR in the culture medium. MMPR concentrations in the medium were not appreciably changed by cellular uptake of MMPR. These data (and those of Table 1) indicated that steady-state concentrations of MMPR-P achieved in the cells were 500 to 600 times higher than the corresponding external MMPR concentrations. Similarly, the entry of natural bases and nucleosides into cells, with subsequent entrapment through conversion to nucleotide derivatives, results in large differences between the concentrations of the external metabolites and their intracellular derivatives. The concentrations of MMPR-P achieved under specified conditions varied somewhat with different cell batches; for example, in 3 experiments (citited below) that involved 2-hr incubations in medium containing 0.5 µM MMPR, cellular concentrations of MMPR-P were 286, 309, and 340 nmoles/10⁹ cells.

When lymphoma cells were incubated for 2 hr in medium containing 0.5 µM MMPR-¹⁴C, the internal concentration of MMPR-P was 340 nmoles/10⁹ cells. A value that did not increase with further incubation. When these cells were transferred to culture medium containing no MMPR, the MMPR-P concentration dropped to 40 nmoles/10⁹ cells during the 1st hr of incubation and by 4 hr had reached 20 nmoles/10⁹ cells; the latter concentration remained unchanged for 19 hr in the absence of MMPR.

These findings imply a turnover of MMPR-P within the L5178Y cell; consistent with this is the further observation that the cellular uptake of 0.5 µM MMPR-¹⁴C during a 1-hr period of incubation was not significantly affected by prior incubation for 2 hr in medium containing 0.5 µM MMPR (unlabeled). The concentrations of labeled MMPR-P were 309 nmoles/10⁹ cells in cells preincubated with MMPR and 286 nmoles/10⁹ cells in cells that were not preincubated.

Purine nucleotide pools changed in size when L5178Y cells were incubated with MMPR. This is apparent in Table 2, in which nucleotide concentrations found in cells after 18 hr of incubation in culture medium containing 0.5 µM MMPR are compared with those in cells incubated without MMPR. Because MMPR treatment did not affect cell size (mean cell volumes in this experiment were 1220 and 1190 cu µm, respectively, for cells cultured with and without MMPR), nucleotide concentrations are expressed as nmoles/10⁹ cells. As seen in Table 2, the presence of 0.5 µM MMPR in the culture medium reduced concentrations of ATP, GTP, and ADP by about 67% and that of UTP by 24%.

Chart 3 represents an attempt to relate cellular MMPR-P concentrations to changes in (a) pool sizes of ribonucleoside triphosphates and (b) proliferation rates during culture of L5178Y cells in MMPR-containing medium. It is seen that, as the cellular content of MMPR-P increased, concentrations of ATP and GTP decreased. When the MMPR-P concentration was 70 nmoles/10⁹ cells, ATP and GTP concentrations were about 60% of control values, but proliferation rates were essentially unchanged. At higher concentrations of MMPR-P, both purine ribonucleotide concentrations and proliferation rates were lower than control values. UTP and CTP concentrations were substantially increased when cells contained MMPR-P; for example, when the cellular MMPR-P concentration was 70
nmoles/10⁹ cells, the UTP concentration was 1.75 times the control value.

Chart 4 shows the effect on nucleotide levels in L5178Y cells of exposure to 0.1 μM MMPR for various periods; this concentration of MMPR did not significantly affect proliferation rates. The intracellular concentration of MMPR-P reached 60 nmoles/10⁹ cells after 1 hr of incubation and stayed at this level throughout the 6-day period. As well, Chart 4 shows that, after 4 hr in MMPR-containing medium, ATP and GTP concentrations were 60 to 70% of control values and stayed at this level for the rest of the experiment. Despite these reductions in purine ribonucleotide concentrations, the cells proliferated at the same rate as controls. Prominent features of these data are the large, MMPR-induced increases in concentrations of UTP and CTP. After 2 hr in MMPR-containing medium, the cellular content of UTP had increased almost 3 times (from 616 to 1713 nmoles/10⁹ cells); subsequently, the UTP concentration fell to about 180% of the control value and remained at that level.

When the lymphoma cells were transferred to MMPR-free medium following incubation for 18 hr in medium containing 0.5 μM MMPR, both nucleotide concentrations and proliferation rates returned to control levels, even though purine nucleotide concentrations had fallen to 30% of control values during MMPR exposure (Chart 5). These results are consistent with the data of Table 3 which show

Table 1

<table>
<thead>
<tr>
<th>MMPR, medium concentration (μM)</th>
<th>MMPR-P, intracellular concentration (μM)</th>
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<tbody>
<tr>
<td>0.05</td>
<td>32</td>
</tr>
<tr>
<td>0.10</td>
<td>60</td>
</tr>
<tr>
<td>0.20</td>
<td>125</td>
</tr>
<tr>
<td>0.30</td>
<td>168</td>
</tr>
<tr>
<td>0.40</td>
<td>229</td>
</tr>
<tr>
<td>0.50</td>
<td>309</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Cells cultured without MMPR</th>
<th>Cells cultured in medium containing 0.5 μM MMPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>597</td>
<td>197</td>
</tr>
<tr>
<td>ATP</td>
<td>1950</td>
<td>683</td>
</tr>
<tr>
<td>UTP</td>
<td>667</td>
<td>827</td>
</tr>
<tr>
<td>CTP</td>
<td>379</td>
<td>341</td>
</tr>
<tr>
<td>ADP</td>
<td>145</td>
<td>50</td>
</tr>
<tr>
<td>NAD</td>
<td>256</td>
<td>160</td>
</tr>
<tr>
<td>MMPR-P</td>
<td>350</td>
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</tr>
</tbody>
</table>
that cell viability, as determined by a cloning assay (6), was not diminished after 18 hr of exposure to 0.5 μM MMPR. Longer exposures to 0.5 μM MMPR greatly reduced cell viability (Table 3).

DISCUSSION

The proliferation of L5178Y cells in culture was prevented by concentrations of MMPR as low as 0.3 μM. MMPR taken up by the cells was converted to the 5'-monophosphate ester, which was the only intracellular metabolite revealed by the methodology used; similar findings with other cells have been reported (2, 4, 12). Presuming that the methyl group of MMPR-P-methyl-14C does not turn over independently of the nucleoside portion of the molecule, the present observation that steady-state concentrations of MMPR-P-methyl-14C occur in these cells after 60 min of incubation with MMPR-methyl-14C indicates that turnover of the intracellular pool of MMPR-P occurs. This conclusion is supported by the following: (a) the cellular content of MMPR-P was reduced to 15% of the initial value by 1 hr of incubation in MMPR-free medium; and (b) preincubation with unlabeled MMPR had little effect on the uptake of labeled MMPR. These findings are in contrast with results from in vivo experiments with Ehrlich ascites tumor cells (20) and human erythrocytes (16) in which it was found that MMPR-P concentrations diminished only slowly. The fate of the disappearing MMPR-P-14C is not known.

The decrease in the purine nucleotide concentrations that occurred when L5178Y cells were incubated with MMPR is in accord with reports by others that MMPR-P is a potent

Chart 3. Relationship between MMPR-P concentrations, nucleoside triphosphate pool sizes, and proliferation rates in cultured L5178Y cells. Cells were incubated for 18 hr in medium containing the indicated concentrations of MMPR-14C. Perchloric acid extracts of the cells were assayed for MMPR-P-14C by paper chromatography and for nucleoside triphosphates by anion-exchange chromatography. Nucleotide concentrations and proliferation rates are expressed as percentages of the same parameters measured in control cells (cultured at the same time in medium without MMPR). O, UTP; □, CTP; ●, GTP; △, ATP; ▲, proliferation rate.

Chart 4. Nucleotide pool sizes in L5178Y cells after various periods of exposure to MMPR. Cultures were incubated for the indicated periods in medium containing 0.1 μM MMPR. Perchloric acid extracts of the cells were assayed for nucleotide concentrations by anion-exchange chromatography. Nucleotide concentrations and proliferation rates are expressed as percentages of those in control cells (cultured at the same time in medium without MMPR). O, UTP; △, CTP; ●, GTP; ■, ATP; □, proliferation rate.

Chart 5. Recovery of L5178Y cells after removal from MMPR-containing medium. Cells were incubated in medium containing 0.5 μM MMPR for 18 hr, at which time cells were transferred to medium without MMPR. Perchloric acid extracts from cell samples taken at the indicated times were analyzed for nucleotide content by anion-exchange chromatography. Nucleotide concentrations and proliferation rates are expressed as percentages of those in control cells (cultured at the same time in medium without MMPR). ●, UTP; ■, CTP; △, ATP; O, GTP; ▲, proliferation rate.
Cells were cultured in medium containing 0.5 μM MMPR for the times noted. Cell viability was determined by the ability of the cells to form colonies in soft agar (6); viability is expressed as a percentage of that for control cells for which plating efficiencies were about 70%.

<table>
<thead>
<tr>
<th>Duration (hr) of MMPR exposure</th>
<th>Viability (%)</th>
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<tbody>
<tr>
<td>18</td>
<td>93</td>
</tr>
<tr>
<td>24</td>
<td>66</td>
</tr>
<tr>
<td>48</td>
<td>40</td>
</tr>
<tr>
<td>72</td>
<td>28</td>
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Inhibitor of PRPP amidotransferase. The ability of L5178Y cells to proliferate was undiminished when purine ribonucleotide concentrations were reduced by as much as 40%. At about 50 to 60% of control values, purine nucleotide concentrations evidently became critical because rates of cell proliferation declined; in cultures with cellular ATP concentrations lower than 30% of control values, proliferation did not occur. Somewhat similar results have been reported in studies on sea urchin eggs (7, 17, 23). In these, decreased mitotic rates were associated with reductions in ATP concentrations; when ATP concentrations were less than 50% of normal values, mitosis was blocked.

L5178Y cells withstood, without loss of viability, 18 hr of exposure to 0.5 μM MMPR, during which time purine ribonucleotide levels fell to 30% of control values. Upon transfer to MMPR-free medium, the MMPR-P concentration fell, purine ribonucleotide concentrations rose, and cell proliferation resumed. Longer exposure to MMPR reduced cell viability. The relationship between MMPR cytotoxicity and effects on purine nucleotide synthesis is unclear because of the possibility that the analog and its metabolites may have additional effects on cell metabolism.

The metabolic basis for the rapid 3-fold increase in concentrations of UTP and CTP that occurred in the presence of 0.1 μM MMPR is uncertain. As PRPP is a substrate in the de novo synthesis of both purine and pyrimidine nucleotides, its availability for the latter process might be enhanced by the MMPR-induced inhibition of purine nucleotide synthesis. However, this explanation does not appear adequate because, in the presence of 0.1 μM MMPR, the increase in UTP and CTP concentrations together represented about 1850 nmoles/10^6 cells, whereas the decrease in ATP and GTP concentrations together amounted to 520 nmoles/10^6 cells. Thus, the PRPP spared by inhibition of purine synthesis does not account for the increased pyrimidine nucleotide concentrations.

**REFERENCES**

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