Abstract—6-Methylmercaptopurine ribonucleoside-5'-phosphate (MeSPuRMP), the sole metabolite of 6-methylmercaptopurine ribonucleoside (MeSPuRib), is a strong inhibitor of purine de novo synthesis, inducing depletion of intracellular purine nucleotides and subsequent cell death in several tumor cell lines. In this study prevention of MeSPuRib cytotoxicity by compounds of the purine salvage pathway was studied in Molt F4 human malignant T-lymphoblasts. Adenosine, adenine and inosine were able to prevent depletion of the adenine nucleotide pool when used in combination with 0.5 µM MeSPuRib, but had virtually no effect on depletion of guanine nucleotides. Nevertheless, these three purine compounds were able to reduce the cytotoxic effects induced by MeSPuRib. Addition of guanosine to cells treated with 0.5 µM MeSPuRib normalized the guanine nucleotide pool, but adenine nucleotides remained depleted. Under these conditions, inhibition of cell growth was significantly decreased. With cell growth and cell viability of Molt F4 cells are less inhibited by MeSPuRib under conditions where adenine nucleotide depletion is prevented by purine compounds (and where the other nucleotides are depleted) we conclude that depletion of adenine nucleotides is an important factor in MeSPuRib cytotoxicity.

MeSPuRib, a adenosine antimetabolite, is cytotoxic for a number of cell lines, and exhibits some anticancer activity in vivo [1-5]. MeSPuRib cytotoxicity is meditated by its metabolite MeSPuRMP, which is formed from MeSPuRib by adenosine kinase (Scheme 1) [3, 6-8]. MeSPuRMP is a strong inhibitor of purine de novo synthesis [9, 10] at PRPP amidotransferase [9, 11-13]. Inhibition of this route induces a depletion of purine nucleotides [4, 5, 14-16], thereby leading to diminution of RNA and DNA formation [14], and subsequent inhibition of cell growth and loss of cell viability [3-5, 15].

MeSPuRMP is also an important metabolite of the anticancer agent 6-MP. 6-MP is first converted into SIMP and the latter into MeSPuRMP by thiopurine methyltransferase [17-19], 6-MP is commonly used in the oral maintenance treatment of children with acute lymphoblastic leukemia [17, 18]. At present it is under discussion whether formation of MeSPuRMP contributes to the anticancer activity of orally administered 6-MP [19]. The metabolic route by which 6-MP is generally thought to induce cytotoxicity is conversion into 6-thioguanine nucleotides, and subsequent incorporation into DNA and RNA [19, 20]. Furthermore, a high activity of thiopurine methyltransferase in red blood cells, resulting in high MeSPuRMP concentrations, correlates with a poor prognosis in children receiving oral 6-MP therapy, suggesting that the methylation route of 6-MP is a catabolic pathway [17, 18]. Our studies of Molt F4 cells, a human malignant lymphoblastic cell line, indicated that under conditions where intracellular MeSPuRMP concentrations were elevated, cytotoxicity of 6-MP was increased [21]. Furthermore, cytotoxicity of both 6-MP and MeSPuRib could be reversed in these cells by addition of adenosine, amidoimidazolecarboxamide ribonucleoside. This compound is converted to AICAR, which is an intermediate of purine de novo synthesis distal to the MeSPuRMP inhibition site [22], providing further evidence for the cytotoxic potency of MeSPuRMP in these cells. These experiments also confirmed that the cytotoxic effect of MeSPuRMP in these cells is the first step in the purine biosynthetic pathway.

In the present study we obtained more evidence regarding MeSPuRMP cytotoxicity in Molt F4 human T-lymphoblasts. To determine whether MeSPuRib cytotoxicity could be prevented by purine intermediates of the purine salvage route (Scheme 1) cell growth, cell viability, endogenous nucleotide
concentrations, extracellular nucleosides and bases, and formation of MeSPuRMP were determined in experiments where cells were treated with various concentrations of MeSPuRib alone, and in combination with adenosine, adenine, inosine or guanosine.

**MATERIALS AND METHODS**

MeSPuRib, adenosine, adenine, inosine and guanosine were obtained from Sigma Chemicals (St Louis, MO, U.S.A.). The experiments were performed with Molt F4 cells, a T-cell acute lymphoblastic leukemia cell line. Conditions for cell culture and experimental procedures have been described earlier [21]. MeSPuRib and adenosine, adenine, inosine, guanosine or combinations of MeSPuRib with one of these purine compounds were added as a single dose in a small volume (1/100).

Intracellular nucleotides (di- and triphosphates) and MeSPuRMP were extracted from 3 × 10⁶ viable cells by means of perchloric acid (PCA, BDH Chemicals Ltd, Poole, U.K.) as described earlier [21] and analysed by means of HPLC at a wavelength of 254 nm and 240 nm, respectively [23]. The concentrations were expressed as pmol/10⁶ viable cells.

Extracellular nucleosides and bases were extracted from 0.5 mL of the culture medium (after the cells had been removed), to which a volume of 25 μL 8 M PCA was added. This was kept on ice for 10 min. Then the samples were centrifuged for 2 min, after which the supernatant was neutralized with 4 M K₂HPO₄. Nucleosides and bases were determined by means of reversed-phase HPLC, with a Supelcosil LC-18-DB column (25 cm × 4.6 mm, Supelco, U.S.A.), and were detected at a wavelength of 254 nm. Concentrations were expressed as μmol/L.

**RESULTS**

Treatment of Molt F4 cells with 0.5 μM MeSPuRib resulted in decreased purine nucleotide concentrations (Table 1) and led to inhibition of cell growth and of cell viability (Fig. 1). The effects of 10 μM MeSPuRib on these parameters were similar (Table 2 and Fig. 2).

Addition of 50 μM adenosine or adenine, or 25 μM inosine in combination with 0.5 μM MeSPuRib prevented the reduction of the intracellular adenine nucleotide pool by MeSPuRib within the first 24 hr of treatment (Table 1). Adenosine was also able to restore the guanine nucleotide pool after 24 hr. Adenine and inosine hardly affected the depletion of guanine nucleotides (Table 1). These purine compounds were able to prevent inhibition of cell growth partially and cell viability nearly completely (Fig. 1). Combination of these purine compounds with 10 μM MeSPuRib partly prevented depletion of the adenine nucleotide pool, especially at 24 hr, but did not prevent the depletion of the guanine nucleotide pool (Table 2). Cytotoxicity was decreased as a result of addition of these purine compounds to treatment with 10 μM MeSPuRib (Fig. 2).

Addition of 25 μM guanosine to treatment with 0.5 μM MeSPuRib resulted in an increase of the intracellular guanine nucleotide pool, but had no effect on the reduction of the adenine nucleotide pool (Table 1). If anything, the reduction of the adenine nucleotide pool became more severe. Furthermore, this combination resulted in almost normal cell viability (Fig. 1b), and cell growth was only partly affected (Fig. 1a). In contrast, guanosine in combination with 10 μM MeSPuRib led to a large
Adenine and guanine nucleotide concentrations (di- and triphosphates) of Molt 4F cells treated with 0.5 μM MeSPuRib alone, or in combination with 50 μM adenosine or adenine, 25 μM inosine or guanosine.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Adenine nucleotides</th>
<th>Guanine nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 μM MeSPuRib</td>
<td>MeSPuRib + Ado</td>
</tr>
<tr>
<td>2</td>
<td>79 (65-95)</td>
<td>111 (95-136)</td>
</tr>
<tr>
<td>6</td>
<td>53 (44-62)</td>
<td>87 (74-88)</td>
</tr>
<tr>
<td>24</td>
<td>47 (36-49)</td>
<td>136 (123-157)</td>
</tr>
<tr>
<td>48</td>
<td>49 (35-55)</td>
<td>47 (40-50)</td>
</tr>
<tr>
<td>72</td>
<td>38 (27-41)</td>
<td>32 (30-37)</td>
</tr>
</tbody>
</table>

Data are expressed as percentages of untreated cells; median and range (between brackets) of three experiments. The adenine nucleotide concentration of Molt 4F cells before treatment is 5180 ± 509 pmol/10^6 viable cells. The guanine nucleotide concentration of Molt 4F cells before treatment is 963 ± 59 pmol/10^6 viable cells.
Table 2. Adenine and guanine nucleotide concentrations (di- and triphosphates) of Molt F4 cells treated with 10 μM MeSPuRib alone, or in combination with 50 μM adenosine or adenine, 25 μM inosine or guanosine.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0.5 μM MeSPuRib</th>
<th>MeSPuRib + Ado</th>
<th>MeSPuRib + Ade</th>
<th>MeSPuRib + Ino</th>
<th>MeSPuRib + Guo</th>
</tr>
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<tbody>
<tr>
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<td>87 (68-96)</td>
<td>73 (66-74)</td>
<td>82 (79-97)</td>
<td>60 (46-70)</td>
</tr>
<tr>
<td>6</td>
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<td>95 (75-106)</td>
<td>71 (60-89)</td>
<td>40 (33-64)</td>
<td>33 (28-43)</td>
</tr>
<tr>
<td>24</td>
<td>57 (50-65)</td>
<td>64 (42-73)</td>
<td>48 (45-54)</td>
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<td>72</td>
<td>36 (30-51)</td>
<td>40 (23-50)</td>
<td>33 (28-43)</td>
<td>27 (22-32)</td>
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</table>

Data are expressed as percentages of untreated cells; median and range (between brackets) of three experiments. The adenine nucleotide concentration of Molt F4 cells before treatment is 5180 ± 509 pmol/10^6 viable cells. The guanine nucleotide concentration of Molt F4 cells before treatment is 963 ± 59 pmol/10^6 viable cells.

**DISCUSSION**

MeSPuRib, an uncharacterized drug, exerts cytotoxic activity after conversion to MeSPuRMP. Inhibitors of methionine adenosyltransferase (MAAT) [8] and purine de novo synthesis [9,10] have been shown to inhibit Molt F4 growth. Addition of guanosine, which inhibits de novo synthesis of guanine nucleotides, resulted in a decrease in viability. Addition of guanosine also led to a slight increase in MeSPuRMP concentration after 24 hr in control experiments.

In a separate experiment, the effects of MeSPuRib on Molt F4 viability were studied in the presence of adenosine or adenine, inosine or guanosine. In both cases, the addition of guanosine resulted in a decrease in viability. However, addition of guanosine also led to a slight increase in MeSPuRMP concentration after 24 hr in control experiments.

MeSPuRib concentrations from the medium were lower than those in the cells, suggesting that the drug is not readily released into the extracellular medium. However, addition of guanosine to the medium resulted in a decrease in viability, indicating that the drug is not readily released into the extracellular medium. Furthermore, addition of guanosine to the medium resulted in a decrease in viability, indicating that the drug is not readily released into the extracellular medium.

**Fig. 2.** Cell growth (a) and cell viability (b) of Molt F4 cells treated with 10 μM MeSPuRib alone, or in combination with 10 μM adenosine, 5 μM adenine, 25 μM inosine or 25 μM guanosine. The results of one experiment are shown. Similar results were obtained in two other experiments.
Table 3. Medium concentrations of purine nucleosides and bases, expressed in µmol/L.

<table>
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<tr>
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</tbody>
</table>

Data from one representative experiment are shown (ND, not detectable).
Fig. 3. MeSPuRMP concentrations of Molt F4 cells treated with either 0.5 µM (a) or 10 µM (b) MeSPuRib alone or in combination with the purine salvage intermediates described in Fig. 1 (expressed as pmoles/10^6 viable cells; mean with standard error of three independent experiments).

**de novo** synthesis [25], these cells are highly susceptible to MeSPuRMP cytotoxicity.

Adenosine, adenine and inosine are able to prevent inhibition of purine **de novo** synthesis induced by 0.5 µM MeSPuRib, leading to almost normal cell growth and cell viability (Fig. 1, Table 1). The effects of these three purine compounds on purine nucleotide concentrations are comparable. The initial normalization during the first 24 hr of intracellular adenine nucleotides is followed by depletion (Table 1). This is the result of the very rapid conversion of the purine bases and nucleosides, as determined by the rapid disappearance of these compounds and their derivates from the incubation medium (Table 3). Reversal of the effects of 3 µM MeSPuRib on induction of differentiation, cell growth inhibition and purine nucleotide concentrations by various concentrations of adenine was observed earlier in HL-60 cells [15] and in sarcoma 180 cells [26]. With 10 µM MeSPuRib normalization of the intracellular adenine nucleotide pool as a result of addition of the purine bases and nucleosides is less pronounced (Table 2). This may be attributed to two phenomena. First, when adenosine is used in combination with a high concentration of MeSPuRib, competition for adenosine kinase may occur, since both adenosine and MeSPuRib are metabolized by this enzyme (Scheme 1). As a result, less adenosine can be phosphorylated by the cells. This process is reflected by the prolonged presence of extracellular hypoxanthine (Table 3), a product of adenosine catabolism, which indicates a slow anabolism of adenosine under these conditions. Second, it is known that a high MeSPuRMP concentration results in inhibition of PRPP synthetase [24, 27, 28]. As PRPP is a substrate for the enzymes adenine
phosphoribosyltransferase and hypoxanthine guan-

cine phosphoribosyltransferase and thus is involved
in the metabolism of adenine and inosine (the latter
being first converted into hypoxanthine), less adenine
and inosine will be incorporated into the cells with
10 μM MeSPuRib. Again, this is reflected by the
concentrations of extracellular adenine and
hypoxanthine after treatment with 10 μM MeSPuRib
in combination with either inosine or adenine (Table 3).

The minor effects of adenine, adenosine and
inosine on the intracellular guanine nucleotide pool
caused by 0.5 μM and 10 μM MeSPuRib (Tables 1 and
2) are probably the result of a preferential
restoration of adenine nucleotide concentration.
Moreover, conversion of bases and nucleosides into
guanine nucleotides is a much slower process than
conversion into adenine nucleotides [29].

The severe cytotoxicity observed with the
combination of 10 μM MeSPuRib and 25 μM
guanosine (Fig. 2) can be ascribed to a nearly
complete reduction in intracellular adenine nucleo-
tides, which is induced by several mechanisms. First,
inhibition of purine de novo synthesis by MeSPuRib
will result in a depletion of adenine nucleotides.
Second, MeSPuRib is converted into MeSPuRMP
by the enzyme adenosine kinase, a reaction which
consumes ATP and thus induces a further decrease
of adenine nucleotide concentrations. Third, addition
of guanosine leads to an increase in guanine
nucleotides 2.5 times that of the control value at
48 hr (Table 2). The formation of GDP and GTP
from GMP consumes ATP by kinase reactions. Therefore
the adenine nucleotide pool will be
depleted further, leading to the dramatic reduction
in intracellular adenine nucleotides observed in these
experiments. Combination of guanosine with 0.5 μM
MeSPuRib does not induce such a severe depletion
of adenine nucleotides (Table 1), since at this
concentration of MeSPuRib less ATP is consumed as
a consequence of the adenosine kinase-mediated conversion of MeSPuRib into MeSPuRMP.
Exaceration of cytotoxicity of MeSPuRib by
guanosine was reported earlier [15, 30, 31], and was
explained by these authors as a synergistic action
between GMP and MeSPuRMP, resulting in a more
severe inhibition of purine de novo synthesis, presumably at PRPP amidotransferase [30]. The results
of our study do not confirm this conclusion, since the guanine nucleotide pools are also elevated with the
combination of 0.5 μM MeSPuRib and guanosine, which does not lead to a more severe
depletion of adenine nucleotides as compared to
0.5 μM MeSPuRib alone (Table 1). Rather the more
severe inhibition of purine de novo synthesis with
the combination of 10 μM MeSPuRib and guanosine
is the result of the severe depletion of the adenine
nucleotide pool observed under these conditions.
Since the conversion of ribose-5'-phosphate to PRPP
is ATP dependent, severe depletion of ATP may
lead to less availability of PRPP for purine de novo
synthesis [24].

The decrease in MeSPuRMP concentrations as a
result of addition of adenosine as compared to
treatment with MeSPuRib alone (Fig. 2) may be the
result of competition between adenosine and
MeSPuRib for adenosine kinase, since both
compounds are metabolized by this enzyme. It is at
present not clear how addition of adenine, inosine
and guanosine affects MeSPuRMP formation.
Perhaps these purine compounds interfere with the
transport of MeSPuRib, thereby decreasing the
concentration of intracellular MeSPuRIB and
MeSPuRMP.

In conclusion, depletion of intracellular adenine
nucleotide concentration appears an important factor
in MeSPuRib cytotoxicity. This is in contrast with
earlier observations that the biological consequences
of purine starvation as a result of MeSPuRib
treatment were primarily due to guanine nucleotide
depression in a mouse T-lymphoma cell line [14]. As
a result of addition of adenosine, adenine and inosine
treatment with MeSPuRib, the adenine nucleotides
were restored to nearly normal values within the
first 24 hr of treatment, whereas guanine nucleotides
remained depleted (Tables 1 and 2), and pyrimidine
nucleotides (data not shown) became depleted as a
result of higher consumption of PRPP by the purine
salvage intermediates. Since cell growth and cell
viability of Molt F4 cells still partly recovered to
control values under these conditions, restoration of
the depletion of adenine nucleotides is also important
for amelioration of the effects of inhibition of purine
de novo synthesis by MeSPuRib.

Furthermore, it appears of importance to use at
least two concentrations of MeSPuRib to study the
effects of purine salvage intermediates on MeSPuRib
induced cytotoxicity, since this reveals more of the
underlying mechanisms of cytotoxicity.

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