Pergamon

Biochemical Pharmacology, Vol. 49, No. 1, pp. 49–56, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00387-4

REVERSAL OF METHYLMERCAPTOPURINE RIBONUCLEOSIDE CYTOTOXICITY BY PURINE RIBONUCLEOSIDES AND ADENINE

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(*Received* 14 *March* 1994; *accepted* 22 *August* 1994)

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 the combination of guanosine and 10 μM MeSPuRib, cytotoxicity was increased compared to 10 μM MeSPuRib alone, associated with a depletion of adenine nucleotides to 9% of untreated cells. Since 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,† an adenosine antimetabolite, is cytotoxic for a number of cell lines, and exhibits some anticancer activity *in vivo* [1–5]. MeSPuRib cytotoxicity is mediated 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 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

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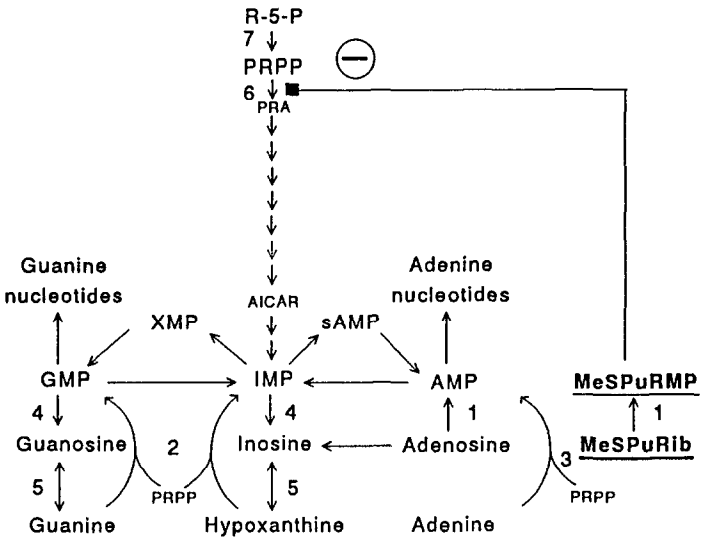
† Abbreviations: MeSPuRMP, 6-methylmercaptopurine ribonucleoside-5′-phosphate; MeSPuRib, 6-methylmercaptopurine ribonucleoside; PRPP, phosphoribosylpyrophosphate; SIMP, 6-thioinosine 5′ monophosphate; 6-MP, 6 mercaptopurine; AICAR, amidoimidazolecarboxamide ribonucleotide; PCA, perchloric acid; IMP, inosine 5′ monophosphate.



Scheme 1. Purine salvage pathway. 1, Adenosine kinase; 2, hypoxanthine guanine phosphoribosyltransferase; 3, adenine phosphoribosyltransferase; 4, 5′ nucleotidase; 5, purine nucleoside phosphorylase; 6, phosphoribosylpyrophosphate amidotransferase; 7, PRPP synthetase.

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 3×10^6 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 pmoles/10^6 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 K_2HPO_4. 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

Table 1. Adenine and guanine nucleotide concentrations (di- and triphosphates) of Molt F4 cells treated with 0.5 μM MeSPuRib alone, or in combination with 50 μM adenosine or adenine, 25 μM inosine or guanosine

	Adenine nucleotides				Guanine nucleotides				
Time (hr)	0.5 μM MeSPuRib	MeSPuRib + Ado	MeSPuRib + Ade	MeSPuRib + Ino	MeSPuRib + Guo	0.5 μM MeSPuRib	MeSPuRib + Ado	MeSPuRib + Ade	MeSPuRib + Ino	MeSPuRib + Guo
2	79 (65–93)	111 (95–126)	90 (81–97)	108 (103–129)	54 (43–56)	63 (57–70)	71 (65–73)	57 (51–67)	80 (75–88)	121 (86–159)
6	53 (44–62)	87 (74–88)	99 (78–102)	99 (80–100)	46 (41–49)	59 (47–60)	62 (61–63)	64 (57–66)	70 (61–75)	290 (257–342)
24	47 (36–49)	136 (123–157)	133 (128–147)	97 (91–107)	60 (55–77)	74 (60–83)	98 (80–109)	72 (70–91)	69 (62–73)	403 (392–484)
48	49 (35–55)	47 (40–50)	48 (45–52)	34 (29–35)	69 (49–110)	65 (59–75)	58 (55–70)	65 (55–68)	62 (55–64)	73 (73–123)
72	38 (27–41)	32 (30–37)	31 (30–33)	27 (25–38)	40 (30–47)	64 (48–75)	69 (56–84)	69 (67–76)	70 (63–81)	52 (50–72)

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 pmoles/10^6 viable cells. The guanine nucleotide concentration of Molt F4 cells before treatment is 963 ± 59 pmoles/10^6 viable cells.

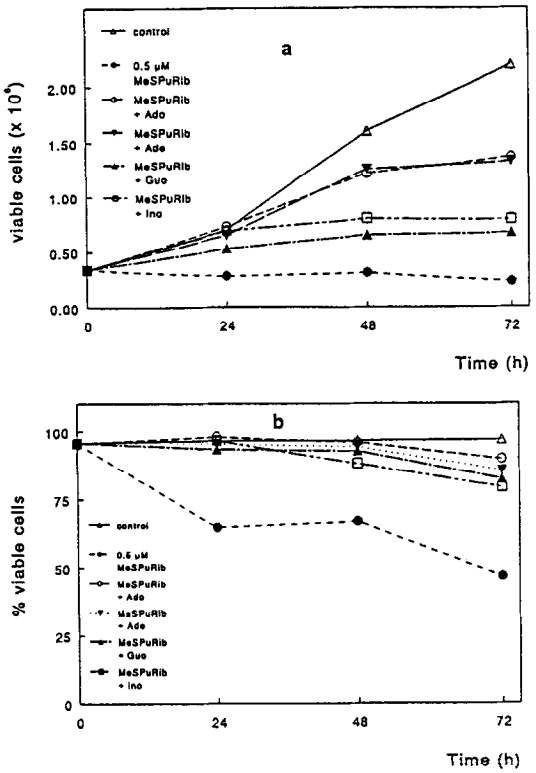


Fig. 1. Cell growth (a) and cell viability (b) of Molt F4 cells after treatment with 0.5 μM MeSPuRib alone or in combination with 50 μM adenosine, 50 μM adenine, 25 μM inosine or 25 μM guanosine. The results of one experiment are shown. Similar results were obtained in two other experiments.

increase of cytotoxicity as compared to 10 μM MeSPuRib alone (Fig. 2). Under these conditions intracellular adenine nucleotides were depleted to 9% of untreated cells after 24 hr (Table 2).

To gain an insight into the metabolic fate of the purine compounds after addition to the cells alone, or in combination with MeSPuRib, concentrations of extracellular nucleosides and bases were determined. The results of these experiments are shown in Table 3. Adenosine can be incorporated via two pathways: directly by adenosine kinase and indirectly by conversion into inosine, which is then converted into hypoxanthine, and subsequently into IMP (Scheme 1). The operation of this second route was reflected by the presence of extracellular inosine and hypoxanthine after addition of 50 μM adenosine to Molt F4 cells (Table 3). No extracellular adenosine was detected. When inosine is used, it is first catabolized to hypoxanthine, which is subsequently phosphorylated by hypoxanthine guanine phosphoribosyltransferase to IMP. Both inosine and hypoxanthine were detectable in the medium for the first 6 hr after the start of the experiment (Table 3). Adenine is metabolized directly by adenine phosphoribosyltransferase to adenine 5′-monophosphate (AMP), and is present in the medium until 48 hr (Table 3). After addition of guanosine alone, both guanosine and guanine are present (Table 3). In general the disappearance of the added

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

Time (hr)	Adenine nucleotides					Guanine nucleotides				
	0.5 μM MeSPuRib	MeSPuRib + Ado	MeSPuRib + Ade	MeSPuRib + Ino	MeSPuRib + Guo	0.5 μM MeSPuRib	MeSPuRib + Ado	MeSPuRib + Ade	MeSPuRib + Ino	MeSPuRib + Guo
2	71 (62–86)	87 (68–96)	73 (66–74)	82 (79–97)	60 (46–70)	57 (50–65)	66 (42–75)	48 (45–54)	64 (60–67)	133 (130–162)
6	38 (31–51)	55 (40–66)	57 (30–58)	61 (49–64)	20 (16–22)	43 (39–60)	42 (35–53)	44 (22–46)	46 (49–49)	209 (153–219)
24	39 (34–45)	90 (84–106)	98 (97–109)	105 (95–122)	9 (6–15)	70 (58–77)	59 (53–68)	52 (49–70)	64 (63–73)	115 (75–189)
48	51 (41–59)	65 (61–80)	71 (70–89)	40 (38–66)	33 (28–42)	70 (60–77)	50 (48–56)	51 (49–54)	49 (46–64)	258 (217–274)
72	50 (40–51)	61 (30–79)	83 (30–96)	32 (27–45)	39 (26–39)	56 (46–63)	57 (36–60)	59 (35–64)	70 (70–90)	217 (155–217)

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 pmoles/10^6 viable cells. The guanine nucleotide concentration of Molt F4 cells before treatment is 963 ± 59 pmoles/10^6 viable cells.

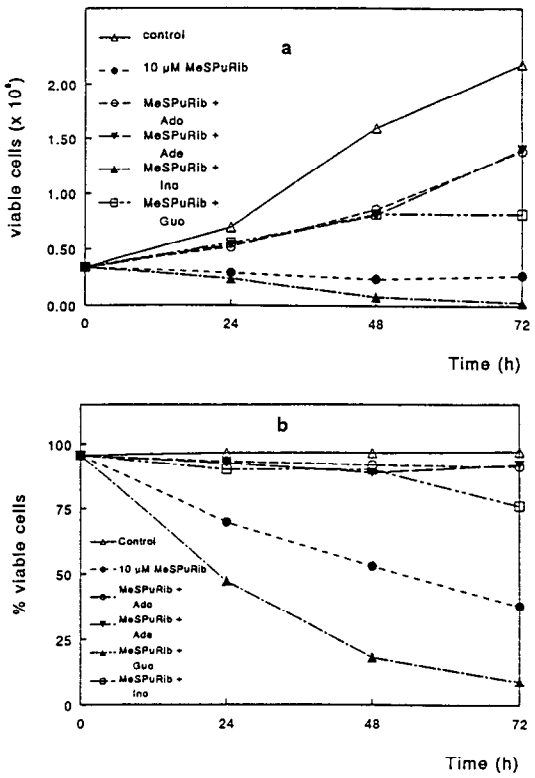


Fig. 2. Cell growth (a) and cell viability (b) of Molt F4 cells after treatment with 10 μM MeSPuRib alone or in combination with 50 μM adenosine, 50 μM adenine, 25 μM inosine or 25 μM guanosine. The results of one experiment are shown. Similar results were obtained in two other experiments.

purine compounds from the medium was slower when added in combination with 10 μM MeSPuRib.

MeSPuRMP concentrations were determined to evaluate the effects of the purine bases and nucleosides on MeSPuRib metabolism. Combination of 0.5 μM MeSPuRib with the purine compounds resulted in a decrease of MeSPuRMP concentrations after 48 hr (Fig. 3a). With 10 μM MeSPuRib, addition of adenosine, adenine and inosine resulted in a decrease in MeSPuRMP concentration. However, addition of guanosine led to a higher MeSPuRMP concentration after 48 hr as compared to 10 μM MeSPuRib alone (Fig. 3b).

DISCUSSION

MeSPuRib, an anticancer drug, exerts its cytotoxic activity after conversion to MeSPuRMP, a strong inhibitor of purine *de novo* synthesis [9, 10]. In this study the effects of MeSPuRib on Molt F4 human T-lymphoblasts have been determined. Inhibition of cell growth, cell viability and purine nucleotide concentrations (Fig. 1, Table 1) are already maximal with 0.5 μM MeSPuRib [24]. With 10 μM MeSPuRib no additional effects on cytotoxicity and on purine nucleotide pools are observed (Fig. 2, Table 2). Because Molt F4 cells have a highly active purine

Table 3. Medium concentrations of purine nucleosides and bases, expressed in μmol/L

	Time (hr)	Hypoxanthine	Adenine	Inosine	Guanosine	Guanine
Control	2	0.39	ND	ND	ND	ND
	6	0.31	ND	ND	ND	ND
	24	0.34	ND	ND	ND	ND
	48	0.49	ND	ND	ND	ND
0.5 μM	2	0.37	ND	ND	ND	ND
MeSPuRib	6	0.33	ND	ND	ND	ND
	24	0.40	ND	ND	ND	ND
	48	0.46	ND	ND	ND	ND
10 μM	2	0.36	ND	ND	ND	ND
MeSPuRib	6	0.37	ND	ND	ND	ND
	24	0.48	ND	ND	ND	ND
	48	0.46	ND	ND	ND	ND
50 μM	2	33.17	ND	13.01	ND	ND
adenosine	6	34.54	ND	4.93	ND	ND
	24	16.03	ND	ND	ND	ND
	48	0.42	ND	ND	ND	ND
50 μM	2	0.67	45.6	ND	ND	ND
adenine	6	0.61	35.11	ND	ND	ND
	24	0.58	15.59	ND	ND	ND
	48	0.44	ND	ND	ND	ND
25 μM	2	18.94	ND	9.62	ND	ND
inosine	6	15.75	ND	1.53	ND	ND
	24	0.53	ND	ND	ND	ND
	48	0.41	ND	ND	ND	ND
25 μM	2	ND	ND	ND	5.40	16.52
guanosine	6	ND	ND	ND	2.45	20.09
	24	0.43	ND	ND	ND	ND
	48	0.37	ND	ND	ND	ND
0.5 μM	2	25.29	ND	14.10	ND	ND
MeSPuRib	6	31.84	ND	4.07	ND	ND
+ adenosine	24	19.33	ND	ND	ND	ND
	48	0.40	ND	ND	ND	ND
0.5 μM	2	0.71	37.36	ND	ND	ND
MeSPuRib	6	0.67	36.32	ND	ND	ND
+ adenine	24	0.68	20.15	ND	ND	ND
	48	0.41	ND	ND	ND	ND
0.5 μM	2	16.43	ND	2.91	ND	ND
MeSPuRib	6	16.42	ND	1.06	ND	ND
+ inosine	24	2.45	ND	ND	ND	ND
	48	0.42	ND	ND	ND	ND
0.5 μM	2	ND	ND	ND	3.50	13.92
MeSPuRib	6	ND	ND	ND	1.40	21.23
+ guanosine	24	ND	ND	ND	ND	6.68
	48	0.39	ND	ND	ND	ND
10 μM	2	26.81	ND	17.59	ND	ND
MeSPuRib	6	34.95	ND	3.35	ND	ND
+ adenosine	24	29.56	ND	ND	ND	ND
	48	13.72	ND	ND	ND	ND
10 μM	2	0.85	39.93	ND	ND	ND
MeSPuRib	6	0.84	38.97	ND	ND	ND
+ adenine	24	1.10	36.29	ND	ND	ND
	48	1.27	14.06	ND	ND	ND
10 μM	2	15.42	ND	3.28	ND	ND
MeSPuRib	6	16.65	ND	0.60	ND	ND
+ inosine	24	8.88	ND	ND	ND	ND
	48	0.56	ND	ND	ND	ND
10 μM	2	ND	ND	ND	4.96	18.04
MeSPuRib	6	ND	ND	ND	1.99	21.93
+ guanosine	24	ND	ND	ND	0.36	18.54
	48	ND	ND	ND	0.47	23.09

Data from one representative experiment are shown (ND, not detectable).

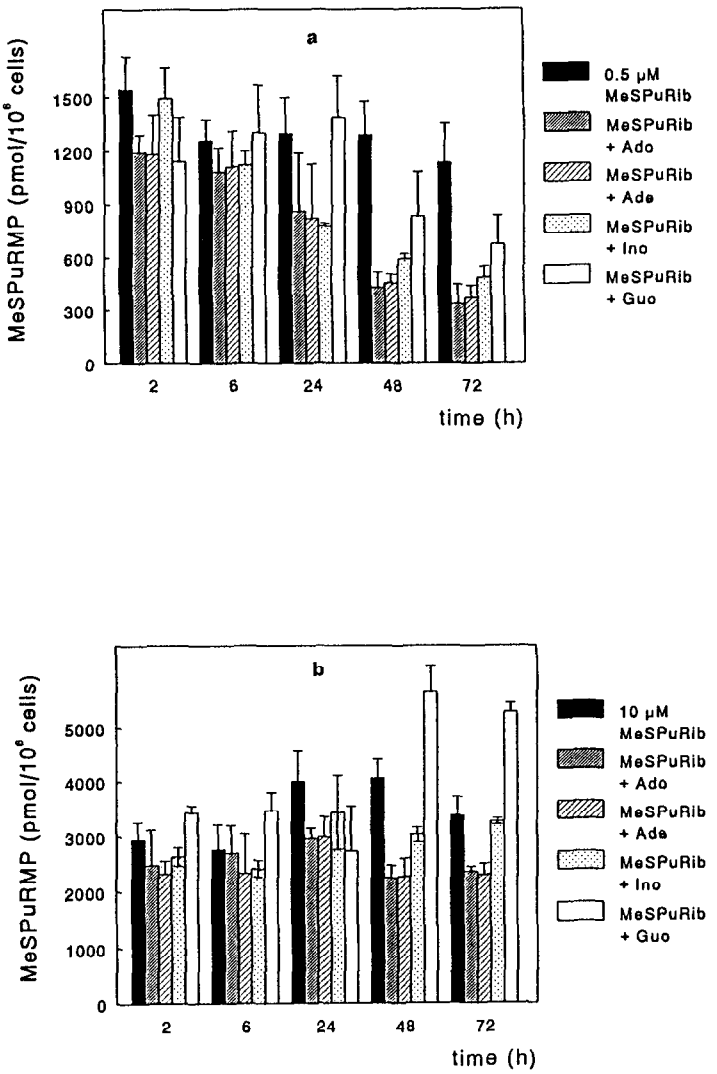


Fig. 3. MeSPuRMP concentrations of Molt F4 cells treated with either 0.5 μM (a) or 10 μM (b) MeSPuRib alone or in combination with the purine salvage intermediates described in Fig. 1 (expressed as pmoles/10^6 viable cells; mean with standard error of three independent experiments).

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 guanine 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 nucleotides, 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. Exacerbation 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 depletion in a mouse T-lymphoma cell line [14]. As a result of addition of adenosine, adenine and inosine to 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.

Acknowledgement—This study was funded by the Dutch Cancer Society, Grant NUKC 89-13.

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