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SEQUENCE-, TIME- AND DOSE-DEPENDENT SYNERGISM OF METHOTREXATE AND 6-MERCAPTOPURINE IN MALIGNANT HUMAN T-LYMPHOBLASTS*

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Abstract—Methotrexate (MTX) and 6-mercaptopurine (6MP) are common drugs in the oral maintenance therapy of acute lymphoblastic leukemia (ALL). On the basis of their biochemical effects on cell metabolism, a sequence-dependent synergism might be anticipated. In order to investigate this hypothesis, MOLT-4 human malignant T-lymphoblasts were incubated with various concentrations of MTX. The time at which maximal increase of intracellular 5-phosphoribosyl-1-pyrophosphate (PRPP) levels was found correlated with the concentrations of MTX used.

Determination of aminoimidazolecarboxamide ribonucleoside monophosphate (AICAR) levels and labeled glycine incorporation into purine metabolites revealed an incomplete inhibition of purine *de novo* synthesis after incubation with 0.02 μM MTX, and a complete inhibition with 0.2 μM MTX.

After prolonged periods of incubation, glutamine exhaustion of the medium caused inhibition of purine *de novo* synthesis in MTX-untreated cells, with a concomitant increase of PRPP levels. Addition of glutamine to the medium prevented this phenomenon.

The increased availability of PRPP after pretreatment with MTX can be used for enhanced intracellular incorporation of hypoxanthine and 6MP in their respective nucleotides. The time- and dose-dependent effects of MTX on PRPP levels correlated with the enhanced incorporation of hypoxanthine and 6MP. The data presented in this study demonstrate that a synergistic action of the combination of MTX and 6MP can be anticipated in malignant lymphoblasts with an active purine *de novo* synthesis depending on the concentration of MTX and on the time and sequence of administration of both drugs.

During the last decade much attention has been paid to drug combinations based on the synergistic and sequence-dependent biochemical effects of the drugs on cell metabolism. The most striking example of synergistic anticancer drug combination is methotrexate (MTX) administration preceding 5-fluorouracil (5FU) [1–8]. Other examples include MTX and 6-thioguanine (6TG) [9], 6-methylmercaptopurine ribonucleoside (MeMPR) and 5FU [1, 10, 11], MeMPR and 6TG [12], and MeMPR and 6-mercaptopurine (6MP) [13–15].

The biochemical basis for these synergistic drug combinations is the inhibition of purine *de novo* synthesis by MTX or MeMPR with concomitant accumulation of 5-phosphoribosyl-1-pyrophosphate (PRPP) [1, 4, 10–12, 14, 16]. The increased availability of PRPP can be used for enhanced conversion of purine and pyrimidine bases and analogues, subsequently administered, and enhanced incorporation into RNA and DNA.

MTX and 6MP have been used for more than 30 years in the therapy of acute lymphoblastic leukemia (ALL) as single drugs [17–20]. The combination of both drugs is in use for more than 20 years [19, 21, 22]. The increased efficacy of the combination in the maintenance therapy of ALL was mainly based on empirical data from studies in patients. To our knowledge no mention has been made in literature of the possibly synergistic action of both drugs, based on the biochemical considerations above. Especially for malignant lymphoblasts with an active rate of purine *de novo* synthesis (Fig. 1), in contrast to normal peripheral blood lymphocytes or normal bone marrow cells without an active purine *de novo* synthesis [23], the synergistic effect should be more dramatic.

In the present study, we first investigated the time- and dose-dependent effect of MTX on the purine *de novo* synthesis in MOLT-4 human malignant T-lymphoblasts, especially with reference to the intracellular PRPP and aminoimidazolecarboxamide ribonucleoside monophosphate (AICAR) levels and the incorporation of labeled glycine. We could demonstrate further that the increased availability of PRPP, due to pretreatment with MTX, can be used for enhanced incorporation of the purine base hypoxanthine and its analogue 6MP. The time at which PRPP levels, and incorporation of hypoxanthine and

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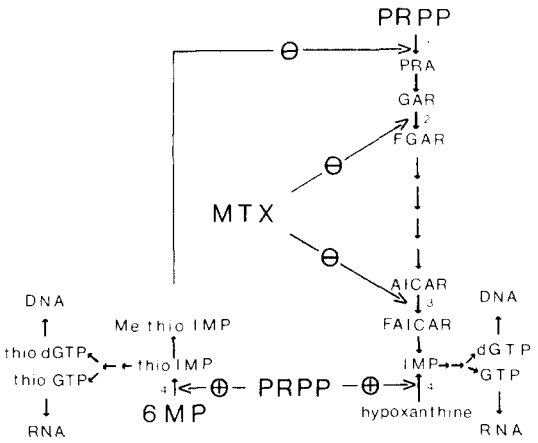

Fig. 1. Simplified scheme of effects of MTX and 6MP on purine metabolism: 1, amidophosphoribosyltransferase (E.C. 2.4.2.14.); 2, glycinamide ribonucleotide formyltransferase (E.C. 2.1.2.2.); 3, aminoimidazolecarboxamide ribonucleotide formyltransferase (E.C. 2.1.2.3.); 4, hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8.).

6MP, are maximal, is correlated with the concentration of MTX used.

Preliminary data of these studies have been reported [24].

MATERIALS AND METHODS

Materials. MTX (Emtrexate PF) was purchased from Pharmachemie (Haarlem, The Netherlands); [carboxyl-^{14}C] orotic acid (51.1 mCi/mmol) from New England Nuclear (Boston, MA); [U-^{14}C] glycine (110 mCi/mmol), [8-^{14}C] hypoxanthine (55 mCi/mmol) and [8-^{14}C] 6-mercaptopurine (1.7 mCi/mmol) from Amersham International Ltd (Amersham, U.K.); PRPP from Sigma (St. Louis, MO); a preparation from brewer's yeast containing orotate phosphoribosyltransferase (OPRT, E.C. 2.4.2.10) and orotidylate decarboxylase (ODC, E.C. 4.1.1.23) from Boehringer Mannheim (Mannheim, F.R.G.); MOLT-4 human T-lymphoblasts from Flow Laboratories (Irvine, U.K.). The E-rosette forming capacity and the presence of T-cell antigens on the MOLT-4 cells in culture was tested every 3 months. The latter by means of monoclonal antibodies [25].

Cell cultures. MOLT-4 cells were allowed to grow at 37° in a water-saturated atmosphere containing 2.5% CO_2 in RPMI medium 1640 Dutch Modification (DM), supplemented with 10% nondialyzed fetal calf serum (v/v), penicillin (100,000 U/l) and streptomycine (100,000 μg/l) in plastic culture flasks. In order to avoid peroxide formation by light in the presence of HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), we supplemented also 2 mM sodium pyruvate [26]. The initial concentration of hypoxanthine in fresh medium, determined by high-performance liquid chromatographic (HPLC) analysis, was 3–5 μM. Logarithmically growing cells were suspended in fresh medium in a concentration of 0.3×10^6 cells/ml 24 hr before each experiment. During the experiments glutamine was added every 24 hr to a final concentration of approximately 2 mM (determined by amino acid analysis) in order to prevent glutamine exhaustion of the medium, which leads to inhibition of purine *de novo* synthesis and concomitant increase of PRPP.

MTX, diluted in medium, was added as a single dose in a small volume (1/100 fraction), and remained in the culture for the duration of the experiment. An appropriate volume of medium was added to untreated cells.

The number of viable cells (trypan blue exclusion) was counted at each point of time in duplicate in a Bürker-Türk chamber.

PRPP assay. We modified and miniaturized the assay for determination of PRPP as described by Peters and Veerkamp [27]. The assay is based on the production of $^{14}CO_2$ from a [carboxyl-^{14}C] orotic acid precursor as described by others [28–30].

An aliquot of 1.0 ml cell suspension was centrifuged (400 *g*, 8 min, 4°), suspended in 125 μl 50 mM Tris–HCl (pH 7.4) containing 1 mM EDTA and lysed by a micro-ultrasonic cell disrupter (Kontes). Inactivation of enzymes was obtained by heating for 45 sec in boiling water. Afterwards correction was made for the destruction of PRPP during this procedure by measuring the recovery of known amounts of PRPP simultaneously. One hundred microliters of the boiled solution were incubated with 10 μl of OPRT/ODC (25 mg/ml) in 100 mM $MgCl_2$ solution and 5 μL (0.8 mM labeled plus 0.8 mM unlabeled) orotic acid in a 0.7 ml reaction vessel (Eppendorf, Hamburg, F.R.G.). This reaction vessel was placed on another empty reaction vessel and subsequently in a polypropylene scintillation minivial containing 0.1 ml ethylene glycol/ethanolamine (2:1 v/v). The minivial was placed in a glass vial sealed with a rubber cap (Fig. 2). In this way all $^{14}CO_2$ liberated was trapped in the ethylene glycol/ethanolamine mixture and the reaction vessel could be removed afterwards without contamination of $^{14}CO_2$. After incubation for 90 min at 37° the reaction was stopped by injection of 40 μl 5 N $HClO_4$ through the rubber cap into the reaction vessel. After a second incubation of 90 min at 37° in order to trap all $^{14}CO_2$ liberated from the reaction mixture, the reaction vessel was removed and 5 ml scintillation fluid was added which consisted of 4 g/l Omnifluor (New England Nuclear, Boston, MA) in Toluene Scintillator

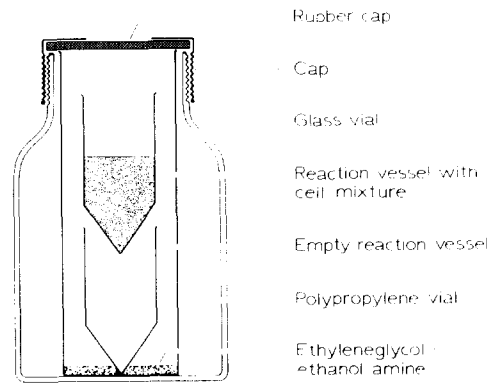

Fig. 2. Micro-assay of PRPP.

(Packard, Groningen, The Netherlands)/methanol (2:1 v/v). The amount of $^{14}CO_2$ trapped in ethylene glycol/ethanolamine was counted in a liquid scintillation analyzer. The total recovery of PRPP standards was 92.8% (SD = 6.1, N = 12). As mentioned before, corrections were made for the destruction of PRPP during heating. The assay is linear between 0.015 nmol and 5.0 nmol (corr. coeff.: 0.9986, P-value: < 0.001, N = 6).

Incorporation of [U-^{14}C] *glycine.* The incorporation of glycine into purine metabolites can be used as a measurement of the activity of purine *de novo* synthesis. We modified the assay described earlier [10]. At intervals indicated, 10 ml of the cell suspension were incubated for 4 hr at 37° with 100 μl [U-^{14}C] glycine. After centrifugation (1350 *g*, 4 min) the cell pellet was washed once with fresh medium in order to remove free glycine. The cell pellet was incubated for 1 hr at 100° with 400 μl 1 N $HClO_4$, in order to precipitate protein and to hydrolyse nucleic acids and nucleotides, which had incorporated glycine. After centrifugation, the pellet was washed with 100 μl 1 N $HClO_4$, and centrifuged again. Both supernatants were neutralized with 4 N KOH, cooled immediately afterwards at −20° and centrifuged. The supernatant was incubated for 1 hr at 37° with 0.3 mg pronase in order to hydrolyse all protein. The samples were evaporated to dryness and redissolved in 25 μl of water. These samples were counted in a liquid scintillation analyzer and further analyzed on two-dimensional thin-layer chromatography, utilizing butanol:methanol:water:ammonia (33%) (30:10:10:1) and secondly 0.15 M NaCl, and using ninhydrin as a marker. This procedure allowed separation of glycine incorporated into adenine, hypoxanthine, guanine and adenosine respectively and free glycine.

After correction for the glycine concentration in fresh medium (determined by amino acid analysis) and correction for losses during the procedure, the amount of glycine incorporated into purine metabolites was calculated and expressed as pmol/hr/10^6 viable cells.

Incorporation of [8-^{14}C] *hypoxanthine and* [8-^{14}C] *6-mercaptopurine.* At various intervals, aliquots of 0.5 ml of the cell culture were sampled and 0.1 ml (570 μM unlabeled plus 30 μM labeled) hypoxanthine was added to a final concentration of 100 μM. After mixing the samples were incubated for 20 min at 37°. The reaction was stopped by cooling (5 min, 0°), cells were centrifuged (8500 *g*, 5 min) and resuspended in 75 μl of the supernatant. These suspensions were layered in a microtube (0.4 ml, Eppendorf, Hamburg, F.R.G.) over a discontinuous gradient consisting of 50 μl 5% saccharose in 0.9% NaCl at the bottom and 70 μl silicone oil (AR 20/AR 200 1:1 v/v, Wacker Chemie, München, F.R.G.) on the top of the gradient. The microtubes were centrifuged (centrifugal force slowly increasing to 7500 *g* and then maintained for 20 sec) in order to separate the cell pellet [31]. The microtubes were cut just above the cell pellet. The cell pellets were resuspended in 2 ml Soluene (Packard, Groningen, The Netherlands) and after 24 hr radioactivity was counted in a liquid scintillation analyzer after addition of 10 ml scintillation fluid: 5% Triton (v/v) in a toluene scintillator.

Analyses with thin layer chromatography after centrifugation of the microtubes showed that the label in the cell pellet after addition of 100 μM [8-^{14}C] hypoxanthine was distributed as follows: 75% ATP, 12% ADP, 3% AMP, 1.2% IMP, 3.6% hypoxanthine, 0.1% adenosine and 5.1% unknown 8-^{14}C label. The saccharose–NaCl solution above the cell pellet did not contain radioactivity. The medium above the oil compartment contained 98% labeled hypoxanthine, 1% labeled inosine and 1% labeled nucleotides but no cells.

Similar experiments were performed at various intervals with [8-^{14}C] hypoxanthine in a final concentration of 10 μM (intracellular label distribution: 82% ATP, 12.2% ADP, 1.7% AMP, 1.0% IMP, 0.4% hypoxanthine and 2.7% unknown), and with [8-^{14}C] 6-mercaptopurine in final concentrations of 100 μM and 10 μM, respectively.

After addition of 100 μM [8-^{14}C] 6-mercaptopurine the intracellular label was distributed as follows: 6.0% ATP, 1.5% ADP (loss of thiol group), 66.6% tIMP, 8.3% thio-inosine, 1.3% 6MP, 11.0% thioguanosine and 5.3% unknown label.

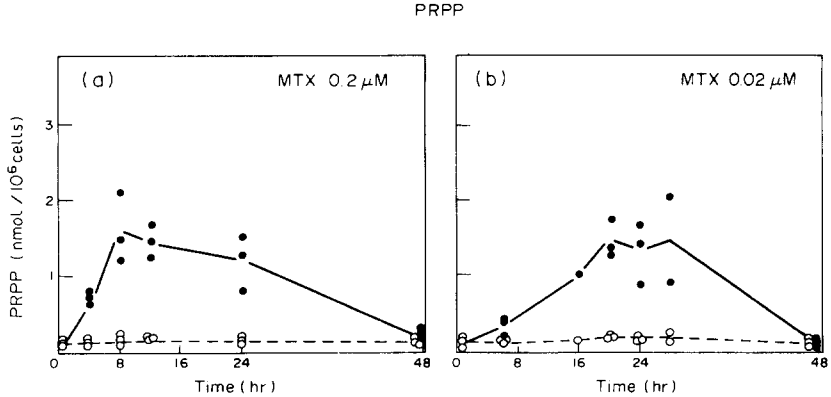

Fig. 3. Effects of MTX on intracellular PRPP levels in MOLT-4 cells. Cells were exposed at $t = 0$ to MTX, and PRPP levels were determined as described in Materials and Methods: (a) 0.2 μM MTX, (b) 0.02 μM MTX. Results were expressed as nmol/10^6 viable cells. ●, MTX-treated cells; ○, untreated cells. Each dot represents a determination in duplicate. The curve is drawn through the mean of the determinations at each point of time.

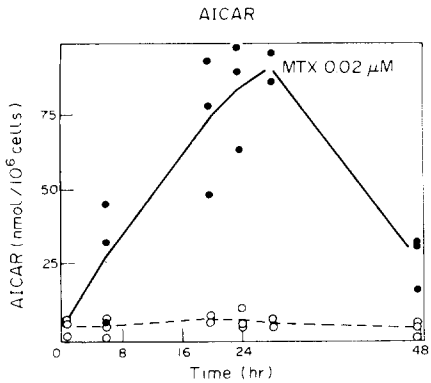

Fig. 4. Effects of MTX on intracellular AICAR levels in MOLT-4 cells. Cells were exposed at $t = 0$ to MTX, and AICAR levels were determined as described in Materials and Methods. Results were expressed as pmol/10^6 viable cells. See further legend for Fig. 3.

The amount of incorporated purine bases was expressed as nmol/10^6 viable cells/20 min.

AICAR assay. Determinations of AICAR were performed by HPLC essentially according to De Abreu *et al.* [32] and the levels were expressed as pmol/10^6 viable cells.

RESULTS

Effects of MTX on PRPP levels

Figure 3 shows the effects on intracellular PRPP levels in MOLT-4 cells after incubation with 0.2 μM and 0.02 μM MTX, respectively. These concentrations of MTX were used, because they can be maintained *in vivo* for many hours after oral administration in the maintenance therapy of ALL in children [33–35]. Concentrations of 0.2 μM MTX increased intracellular PRPP levels 11-fold after 8 hr of incubation, whereas 0.02 μM MTX increased PRPP levels 9-fold with a maximum reached at a later point of time (i.e. between 20 and 28 hr). After 48 hr PRPP levels decreased to those of untreated cells.

Effects of MTX on AICAR levels and [U-^{14}C] *glycine incorporation*

After incubation of MOLT-4 cells with 0.02 μM MTX a significant (18-fold) increase of AICAR was observed with a maximum at 28 hr, followed by a decrease at 48 hr (Fig. 4), suggesting an incomplete inhibition of purine *de novo* synthesis at 20–28 hr, which recovered at 48 hr. Incubations with 0.2 μM MTX (data not shown) did not show any increase of AICAR in comparison with untreated cells, suggesting a complete inhibition. The results of glycine incorporation into purine metabolites (Table 1) indicate a complete inhibition of purine *de novo* synthesis at 24 and 48 hr after incubation with 0.2 μM MTX, whereas treatment with 0.02 μM MTX did not result in a significant inhibition.

Effects of MTX on intracellular incorporation of [8-^{14}C] *hypoxanthine and* [8-^{14}C] *6-mercaptopurine*

Figures 5a and 5b represent the incorporation for 20 min of 100 μM hypoxanthine at various points of time after incubation with 0.2 μM and 0.02 μM MTX, respectively.

Table 1. Incorporation of [U-^{14}C] glycine* in MOLT-4 cells exposed to MTX

MTX (μM)	Duration of exposure 24 hr	48 hr
0	214 ± 38 (10)	231 ± 137 (4)
0.02	189 ± 100 (8)	219 ± 71 (4)
0.2	0 (4)	0 (4)

* In pmol/hr/10^6 viable cells ± SD.
Numbers in parentheses: number of experiments.

Figures 5c and 5d represent the same data after incorporation of 10 μM hypoxanthine.

Similar curves were observed as compared to those of intracellular PRPP levels (Fig. 3).

Incorporation (for 20 min) with [8-^{14}C] 6-mercaptopurine in a concentration of 100 μM or 10 μM at various points of time after incubation with MTX is demonstrated in Figs. 6a–6d. Again similar curves were observed as compared to Figs. 3 and 5.

DISCUSSION

Methotrexate is known to inhibit dihydrofolate reductase (DHFR). The consequences of inhibition of DHFR include accumulation of dihydrofolates and corresponding depletion of tetrahydrofolates. The results are an inhibition of thymidylate synthetase in pyrimidine biosynthesis and inhibition of tetrahydrofolate dependent enzymes in purine *de novo* synthesis with concomitant increase of intracellular PRPP levels [4, 16]. Figure 3 demonstrates the correlation between the period of time after which PRPP levels are maximal in MOLT-4 T-lymphoblasts and the concentrations of MTX used. We found that 0.002 μM MTX did not result in any increase of PRPP in a time period of 48 hr (data not shown), 0.02 μM MTX resulted in a peak of PRPP at 20–28 hr, 0.1 μM MTX in a peak after 18 hr (data not shown) and 0.2 μM MTX in a peak 8 hr after incubation with MTX. Thus, higher concentrations of MTX resulted in earlier peak levels of intracellular PRPP. The correlation between the concentration of MTX and the moment of maximal PRPP increase seems of importance in combination chemotherapy consisting of MTX and those antimetabolites which use PRPP as a cofactor for their phosphorylation and which are ultimately incorporated into RNA and DNA.

We could demonstrate the importance of glutamine as an essential nutrient in cells with an active purine *de novo* synthesis as mentioned by others [36, 37]. When we omitted glutamine addition every 24 hr, glutamine exhaustion of the medium resulted in an inhibition of purine *de novo* synthesis with concomitant increase of PRPP, especially in untreated MOLT-4 cells, because glutamine is a cosubstrate of amidophosphoribosyltransferase (Fig. 1). The high capacity of purine *de novo* synthesis in MOLT-4 cells is reflected in the high PRPP levels in untreated cells: 0.100 ± 0.045 nmol/10^6 viable cells (mean ± SD, 12 experiments). This is in contrast

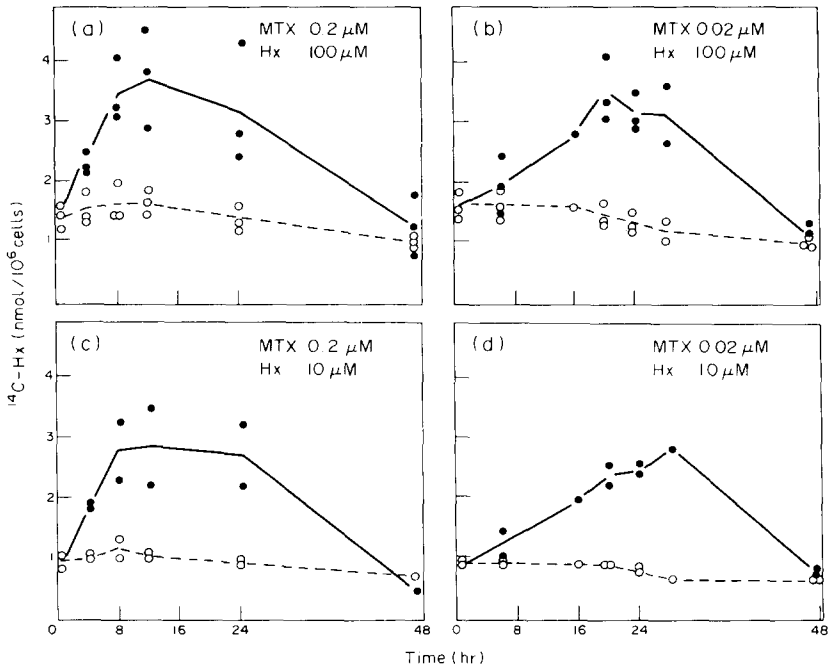

Fig. 5. Effects of MTX on intracellular incorporation of [8-^{14}C] hypoxanthine in MOLT-4 cells. Cells were exposed at $t = 0$ to MTX. At each point of time indicated, 0.5 ml of the cell suspension was incubated during 20 min with [8-^{14}C] hypoxanthine and intracellular incorporation was determined as described in Materials and Methods. Results were expressed as nmol/10^6 viable cells/20 min; (a) 0.2 μM MTX, 100 μM hypoxanthine; (b) 0.02 μM MTX, 100 μM hypoxanthine; (c) 0.2 μM MTX, 10 μM hypoxanthine; (d) 0.02 μM MTX, 10 μM hypoxanthine. See further legend for Fig. 3.

with the low PRPP levels in normal, nonstimulated peripheral blood lymphocytes: 0.008 nmol/10^6 cells [38]. We could not demonstrate a significant increase of PRPP in normal peripheral blood lymphocytes after incubation with 0.02 and 0.2 μM MTX over a time period of 48 hr (data not shown) and we could not demonstrate cytotoxicity. These data suggest that leukemic lymphoblasts are more susceptible to the biochemical disturbances of purine *de novo* synthesis after treatment with MTX in comparison with

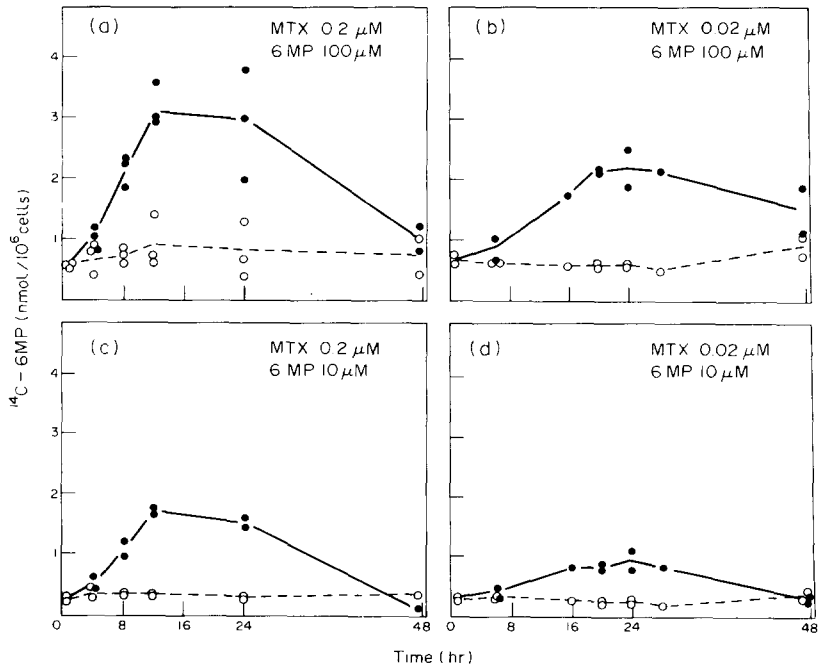

Fig. 6. Effects of MTX on intracellular incorporation of [8-^{14}C] 6-mercaptopurine in MOLT-4 cells; (a) 0.2 μM MTX, 100 μM 6MP; (b) 0.02 μM MTX, 100 μM 6MP; (c) 0.2 μM MTX, 10 μM 6MP; (d) 0.02 μM MTX, 10 μM 6MP. See further legends for Figs. 5 and 3.

normal peripheral blood lymphocytes and also in comparison with normal bone marrow cells which did not show an active purine *de novo* synthesis as well [23].

The two enzymes, involved in inhibition of purine *de novo* synthesis by MTX, are glycinamide ribonucleotide (GAR) formyltransferase and aminoimidazolecarboxamide ribonucleotide (AICAR) formyltransferase (Fig. 1). The slow increase of PRPP and AICAR (Figs. 3 and 4) with maxima at 20–28 hr after incubation with 0.02 μM MTX demonstrates that at this concentration of MTX GAR-formyltransferase is only partially inhibited and that the inhibition of AICAR-formyltransferase is more pronounced. However, even the latter enzyme might also be partially inhibited. After 48 hr AICAR levels recovered, suggesting a recovery from the partial inhibition of purine *de novo* synthesis. The fact that we did not find a significant inhibition of glycine incorporation after 24 hr (Table 1) may be due to the excess of glycine under the experimental conditions in the assay.

Incubation with 0.2 μM MTX did not increase AICAR levels, which suggests a complete inhibition of GAR-formyltransferase with higher concentrations of MTX. This was confirmed by glycine incorporation experiments (Table 1) and by the changes in intracellular ribonucleotide pools [39]. The decline of PRPP levels in time is due to reutilization of nucleotide precursors. This will be explained in more detail in the accompanying paper [39].

The increased availability of PRPP due to MTX pretreatment can be used for increased conversion and incorporation of natural purine and pyrimidine bases. This was confirmed in our incorporation studies with [8-^{14}C] hypoxanthine. The amounts of incorporated hypoxanthine are directly correlated with PRPP levels at each point of time after incubation with MTX (Figs. 5 and 3). The results of incorporation with 10 μM hypoxanthine are especially important, because the initial concentration of hypoxanthine in the medium was 3–5 μM (see below). These data support the findings of others, that natural purine and pyrimidine bases can be used in order to rescue a prolonged purineless and thymidylateless state after treatment with MTX [6, 16, 40–45].

Figure 6 demonstrates that the increased availability of PRPP due to pretreatment of MTX can also be used for enhanced incorporation of 6-mercaptopurine. Again, the curves are similar to those of intracellular PRPP levels (Fig. 3) and those of incorporation of hypoxanthine (Fig. 5) and emphasize the correlation between the concentration of MTX and the moment of maximal 6MP incorporation.

The amount of 6MP incorporated in untreated cells is approximately 2–3 times lower than the amount of hypoxanthine incorporated. This can be explained by the fact that hypoxanthine can be incorporated into nucleic acids in the form of both adenine and guanine (deoxy)ribonucleotides with a preference for adenine [31]. 6MP, however, can only be incorporated as thioguanine (deoxy)ribonucleotides. The results of incorporation with 10 μM 6MP indicate that this concentration is also able to show an increased incorporation, i.e. conversion by HGPRT, whereas the initial concentration of hypoxanthine in the medium is 3–5 μM. Hypoxanthine is the physiologic substrate for HGPRT and is competitive with 6MP for this enzyme. However, it should be mentioned that treatment with MTX causes depletion of purine metabolites and that the increase of PRPP will be utilized first for conversion of hypoxanthine, present in the medium, before 6MP is added. We could demonstrate a decrease of the initial concentration of hypoxanthine in the medium of 3–5 μM to 0.1–0.2 μM after 24 and 48 hr (data not shown). Nevertheless, after consumption of PRPP by hypoxanthine in the medium there was still a significant increase of PRPP levels (Fig. 3), which now became available for conversion of 6MP by HGPRT. These phenomena are of special importance in the *in vivo* situation, where the concentration of hypoxanthine is variable [46]. These phenomena indicate that the concentration of 6MP must exceed the actual concentration of hypoxanthine in order to obtain a significant intracellular incorporation of 6MP. Our results indicate that pretreatment with MTX lowers the hypoxanthine concentration of the medium. So, pretreatment of MTX potentiates also the conversion of 6MP by lowering its competitive substrate for HGPRT. Although we found hypoxanthine levels in bone marrow samples of 4.8 ± 2.1 μM (mean ± SD, N = 11), which are similar to those of our experimental conditions, this phenomenon has to be proven for the *in vivo* situation.

The data presented in this study demonstrate that a synergistic action of the combination of MTX and 6MP can be anticipated depending on the concentration of MTX and on the time and sequence of administration of both drugs. At present, we investigate the sequence-, time- and dose-dependent effects of various combinations of MTX and 6MP in several human malignant lymphoblastic cell lines with respect to purine *de novo* synthesis, availability of PRPP and cytotoxicity (soft agar colony forming activity).

The synergistic action of MTX and other purine and pyrimidine base analogues administered after MTX has been demonstrated in many *in vitro* studies: MTX and 5FU [1, 2, 4, 6] and MTX and 6TG [9]. Under the experimental conditions used, these studies also revealed that the analogues preferentially incorporate into RNA, whereas DNA synthesis is inhibited by MTX. However, the differences in time of maximal PRPP levels and maximal incorporation of the analogues in relation to various concentrations of MTX have not been referred. Studies are in progress in our laboratory investigating the incorporation of 6MP with and without pretreatment of MTX into newly formed DNA and RNA, by means of double-labeling techniques with ^{14}C-6MP and ^{32}P.

MTX and 6MP are common drugs in the maintenance therapy of children with ALL. Both drugs are administered orally. The bioavailability of MTX after oral administration differs from patient to patient [33–35, 47]. However, the serum concentrations of MTX range around levels which have been used in our studies. The bioavailability of 6MP after oral administration is extremely limited and

variable [48–51]. Moreover, because of the complex metabolism of 6MP an evaluation of the pharmacokinetics and bioavailability of 6MP will be very difficult.

Nevertheless, further pharmacokinetic studies in children with ALL and *in vitro* experiments in lymphoblastic cell lines on the combination of MTX and 6MP are necessary in order to elucidate whether the present oral administration of MTX and 6MP in the maintenance therapy of ALL is sufficient to take an optimal advantage of the possible synergism of both drugs as presented in this study.

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REFERENCES

1. C. Benz and E. Cadman, *Cancer Res.* **41**, 994 (1981).
2. J. R. Bertino, W. L. Sawicki, C. A. Lindquist and V. S. Gupta, *Cancer Res.* **37**, 327 (1977).
3. G. P. Browman, *Cancer Treat. Rep.* **68**, 465 (1984).
4. E. Cadman, R. Heimer and L. Davis, *Science* **205**, 1135 (1979).
5. B. A. Chabner, *Cancer* **54**, 2599 (1984).
6. A. A. Piper, S. E. Nott, W. B. Mackinnon and M. H. N. Tattersall, *Cancer Res.* **43**, 5701 (1983).
7. S. W. Pitman, C. D. Kowal and J. R. Bertino, *Semin. Oncol.* **10**, (suppl. 2) 15 (1983).
8. U. Ringborg, G. Ewert, J. Kinnman, P.-G. Lundquist and H. Strander, *Semin. Oncol.* **10**, (suppl. 2) 20 (1983).
9. R. D. Armstrong, R. Vera, P. Snyder and E. Cadman, *Biochem. biophys. Res. Comm.* **109**, 595 (1982).
10. E. Cadman, C. Benz, R. Heimer and J. O'Shaughnessy, *Biochem. Pharmac.* **30**, 2469 (1981).
11. W. P. Peters, G. Weiss and D. W. Kufe, *Cancer Chemother. Pharmac.* **13**, 136 (1984).
12. J. A. Nelson and R. E. Parks, Jr., *Cancer Res.* **32**, 2034 (1972).
13. A. R. P. Paterson and A. Moriwaki, *Cancer Res.* **29**, 681 (1969).
14. A. R. P. Paterson and M. C. Wang, *Cancer Res.* **30**, 2379 (1970).
15. E. M. Scholar, P. R. Brown and R. E. Parks, Jr., *Cancer Res.* **32**, 259 (1972).
16. J. M. Buesa-Perez, A. Leyva and H. M. Pineda, *Cancer Res.* **40**, 139 (1980).
17. J. H. Burchenal, M. L. Murphy, R. R. Ellison, M. P. Sykes, T. C. Tan. L. A. Leone, D. A. Karnofsky, L. F. Craver, H. W. Dargeon and C. P. Roads, *Blood* **8**, 965 (1953).
18. S. Farber, L. K. Diamond, R. D. Mercer, R. F. Sylvester, Jr. and J. A. Wolff, *N. Engl. J. Med.* **238**, 787 (1948).
19. E. Frei III, E. J. Freireich, E. Gehan, D. Pinkel, J. F. Holland, O. Selawry, F. Haurani, C. L. Spurr, D. M. Hayes, G. W. James, H. Rothberg, D. B. Sodee, R. W. Rundles, L. R. Schroeder, B. Hoogstraten, I. J. Wolman, D. G. Traggis, T. Cooper, B. R. Gendel, F. Ebaugh and R. Taylor, from the Acute Leukemia Group B, *Blood* **18**, 431 (1961).
20. E. J. Freireich, E. Gehan, E. Frei, III, L. R. Schroeder, I. J. Wolman, R. Anbari, E. O. Burgert, S. D. Mills, D. Pinkel, O. S. Selawry, J. H. Moon, B. R. Gendel, C. L. Spurr, R. Storrs, F. Haurani, B. Hoogstraten and S. Lee, from the Acute Leukemia Group B, *Blood* **21**, 699 (1963).
21. E. Frei, III and S. E. Sallan, *Cancer* **42**, 828 (1978).
22. D. Pinkel, *Cancer* **43**, 1128 (1979).
23. H. Becher, M. Weber and G. W. Lohr, *Klin. Wschr.* **56**, 274 (1978).
24. J. P. M. Bökkerink, T. J. Schouten, R. A. De Abreu, R. J. J. Lippens, G. A. M. de Vaan, C. H. M. M. de Bruyn and J. P. R. M. van Laarhoven, *T. Kindergeneesk.* **52** 118 (1984).
25. A. Polliack, H. Gamliel, H. B. Bassat, D. Gurfel, R. Leizerowitz and J. Minowada, *Cancer* **51**, 72 (1983).
26. G. T. Spierenburg, F. T. J. J. Oerlemans, J. P. R. M. van Laarhoven and C. H. M. M. de Bruyn, *Cancer Res.* **44**, 2253 (1984).
27. G. J. Peters and J. H. Veerkamp, *Int. J. Biochem.* **10**, 885 (1979).
28. S. R. May and R. S. Krooth, *Analyt. Biochem.* **75**, 389 (1976).
29. G. H. Reem, *Science* **190**, 1098 (1976).
30. W. J. M. Tax and J. H. Veerkamp, *Clin. chim. Acta* **78**, 209 (1977).
31. M. M. Müller, G. Pischek, O. Scheiner, H. Stemberger and G. Wiedermann, *Blut* **38**, 447 (1979).
32. R. A. De Abreu, J. M. van Baal, T. J. Schouten, E. D. A. M. Schretlen and C. H. M. M. de Bruyn, *J. Chromatogr.* **227**, 526 (1982).
33. F. M. Balis, J. L. Savitch and W. A. Bleyer, *Cancer Res.* **43**, 2342 (1983).
34. A. W. Craft, A. Rankin and W. Aherne, *Cancer Treat. Rep.* **65**, (suppl. 1) 77 (1981).
35. C. R. Pinkerton, S. G. Welshman, J. G. Kelly, R. G. Shanks and J. M. Bridges, *Cancer Chemother. Pharmac.* **10**, 36 (1982).
36. W. Engström and A. Zetterberg, *J. Cell. Physiol.* **120**, 223 (1984).
37. H. R. Zielke, C. L. Zielke and P. T. Ozand, *Fedn. Proc. Fedn Am. Soc. exp. Biol.* **43**, 121 (1984).
38. G. J. Peters, A. Oosterhof and J. H. Veerkamp, *Int. J. Biochem.* **13**, 577 (1981).
39. J. P. M. Bökkerink, R. A. De Abreu, M. A. H. Bakker, M. W. Hulscher, J. M. van Baal and G. A. M. De Vaan, *Biochem. Pharmac.* **35**, 000 (1986).
40. G. P. Browman, *Cancer Treat. Rep.* **66**, 2051 (1982).
41. W. M. Hryniuk, *Cancer Res.* **35**, 1085 (1975).
42. W. M. Hryniuk, *Cancer Res.* **35**, 1427 (1975).
43. A. Leyva, H. Nederbragt, J. Lankelma and H. M. Pinedo, *Cancer Treat. Rep.* **65**, (suppl. 1) 45 (1981).
44. H. M. Pinedo, D. S. Zaharko, J. M. Bull and B. A. Chabner, *Cancer Res.* **36**, 4418 (1976).
45. I. W. Taylor, P. Slowiaczek, P. R. Francis and M. H. N. Tattersall, *Molec. Pharmac.* **21**, 204 (1982).
46. S. B. Howell, K. Herbst, G. R. Boss and E. Frei, III, *Cancer Res.* **40**, 1824 (1986).
47. B. A. Kamen, J. S. Holcenberg, K. Turo and V. M. Whitehead, *J. Pediatr.* **104**, 131 (1984).
48. S. Zimm, J. M. Collins, R. Riccardi, D. O'Neill, P. K. Narang, B. Chabner and D. G. Poplack, *N. Engl. J. Med.* **308**, 1005 (1983).
49. T. J. Schouten, R. A. De Abreu, E. D. A. M. Schretlen, J. M. van Baal, M. B. van Leeuwen and G. A. M. de Vaan, in *6-Mercaptopurine and 6-Thioguanine: A Fresh Look on Vintage Anticancer Drugs* (Thesis, T. J. Schouten), p. 87. Stichting Studenttenpers, Nijmegen (1985).
50. L. Lennard, C. A. Rees, J. S. Lilleyman and J. L. Maddocks, *Br. J. clin. Pharmac.* **16**, 359 (1983).
51. T. J. Schouten, R. A. De Abreu, C. H. M. M. De Bruyn, E. Van Der Kleijn, J. M. Oosterbaan, E. D. A. M. Schretlen and G. A. M. De Vaan, *Adv. exp. Med. Biol.* **165B**, 367 (1984).