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PURINE *DE NOVO* SYNTHESIS AS THE BASIS OF SYNERGISM OF METHOTREXATE AND 6-MERCAPTOPURINE IN HUMAN MALIGNANT LYMPHOBLASTS OF DIFFERENT LINEAGES*

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Abstract—Methotrexate (MTX) causes an inhibition of purine *de novo* synthesis (PDNS), resulting in increased intracellular availability of 5-phosphoribosyl-1-pyrophosphate (PRPP) in human malignant lymphoblasts with an active PDNS. Normal bone marrow cells and peripheral blood lymphocytes lack this capacity. The increased levels of PRPP can be used for enhanced incorporation of 6-mercaptopurine (6MP), indicating a potential time-, sequence- and dose-dependent synergism of both drugs. The effects of 0.02 μM and 0.2 μM MTX on the PDNS of MOLT-4 (T-), RAJI (B-) and KM-3 (non-B-non-T-) human malignant lymphoblasts were studied with respect to PRPP levels, aminoimidazolecarboxamide ribonucleosidemonophosphate (AICAR) levels and the incorporation of labeled glycine into purine metabolites. These results were correlated with the activity of the PDNS (labeled glycine incorporation) and the purine salvage pathway (labeled hypoxanthine incorporation) in untreated cells. Inhibition of PDNS by 0.02 μM MTX was complete in KM-3 cells with a moderately active PDNS and salvage pathway. RAJI cells, with a relatively low PDNS and high salvage pathway, demonstrated an incomplete, but increasing inhibition of PDNS, whereas inhibition of PDNS in MOLT-4 cells with both pathways active was minimal and recovered in time. Treatment with 0.2 μM MTX resulted in a complete inhibition of PDNS in all cell lines. After treatment with MTX an enhanced incorporation of labeled hypoxanthine and 6MP was noticed, confirming the potential rescue from MTX cytotoxicity by hypoxanthine and a potential synergism of MTX and 6MP on cytotoxicity. The enhanced incorporation of 6MP was more obvious in RAJI and KM-3 cells in comparison with MOLT-4 cells. These data demonstrate the important role of both the activities of the PDNS and the purine salvage pathway in malignant lymphoblasts of different subclasses with respect to the synergism of MTX and 6MP.

During the last years much attention has been paid to key enzymes in the purine salvage pathway and interconversion in B- and T-lymphocytes and leukemic lymphoblasts. Congenital deficiencies of adenosine deaminase (ADA, EC 3.5.4.6.), purine nucleoside phosphorylase (PNP, EC 2.4.2.1.) and 5'nucleotidase (5'NT, EC 3.1.3.5.) are associated with severe disturbances in lymphocyte-mediated immune function. These and other key enzymes play an important role in lymphoid cell differentiation [1, 2]. The differences in purine enzyme activities in malignant lymphoblasts can be used as diagnostic biochemical markers in immunologically different subclasses of acute lymphoblastic leukemia (ALL) [3–7]. Moreover, they may serve as specific targets for chemotherapy with specific inhibitors; for example: high activities of ADA in T-ALL can be inhibited by 2' deoxycoformycin, leading to cell kill [8, 9]. Otherwise, alterations in enzyme activities may result in resistance to certain chemotherapeutic agents; for example hypoxanthineguanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8.) deficiency due to somatic mutations is associated with resistance to 6-mercaptopurine (6MP), 6-thioguanine (6TG) and azathioprine [10, 11].

Methotrexate (MTX) and 6MP have been utilized for many years in the oral maintenance therapy of ALL [12]. The increased therapeutic efficacy of the combination of these drugs was based on empirical data from studies in mice [13] and in patients [14]. However, on the basis of their interactions with the purine *de novo* synthesis (PDNS) and the purine salvage pathway, a synergism of both drugs could be expected.

We demonstrated a sequence-, time-, and dose-dependent synergism of MTX and 6MP in MOLT-4 human malignant T-lymphoblasts [15]. We studied the effects of MTX on the PDNS of MOLT-4 cells, with special reference to intracellular 5-phosphoribosyl-1-pyrophosphate (PRPP) and aminoimidazolecarboxamide ribonucleosidemonophosphate (AICAR) levels and the incorporation of labeled glycine. The increased availability of PRPP after pretreatment with MTX could be used for an enhanced intracellular conversion of 6MP. The time at which the inhibition of PDNS and the incorporation of 6MP were maximal depended on the concentration of MTX. The concentrations of MTX

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used in these studies (0.02 μM and 0.2 μM) can be maintained *in vivo* for many hours in the oral maintenance therapy of ALL in children [16–18].

Although the role of the purine salvage pathway in various subclasses of ALL has been well established as mentioned above, scarce data have been published with respect to the role of the PDNS in these cells and in normal lymphocytes and bone marrow cells [19]. In the present study, we compare the time- and dose-dependent effects of MTX on the PDNS and on the intracellular incorporation of labeled hypoxanthine (Hx) and its analogue 6MP in the three human malignant lymphoblastic cell lines: MOLT-4 (T-cells), RAJI (B-cells), and KM-3 (common ALL, non-B-non-T-cells).

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 phosphoribosyl transferase (OPRT, EC 2.4.2.10) and orotidylate decarboxylase (ODC, EC 4.1.1.23) from Boehringer Mannheim (Mannheim, F.R.G.). The cell lines MOLT-4 and RAJI have been maintained in continuous culture in our laboratory for several years. KM-3 cells were supplied by the Department of Hematology, St Radboud Hospital, University of Nijmegen (head prof. Dr. C. Haanen). The absence of mycoplasma contamination, the E-rosette forming capacity and the presence of antigens on the cells in culture were tested regularly. The latter by means of monoclonal antibodies [20].

Cell cultures. All cell cultures 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% non-dialyzed fetal calf serum (v/v), penicillin (100,000 U/L), streptomycine (100,000 μg/L), and sodium pyruvate (2 mM) in plastic culture flasks. Logarithmically growing cells were resuspended 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 in order to avoid glutamine exhaustion of the medium [15].

MTX, diluted in medium, was added as a single dose in a small volume (1/100 fraction) in a final concentration of 0.02 μM and 0.2 μM, respectively, 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 and incorporation of [U-^{14}C] *glycine.* The PRPP assay was based on the production of $^{14}CO_2$ from a [carboxyl-^{14}C] orotic acid precursor, as described previously by us [15]. The incorporation of [U-^{14}C] glycine into purine metabolites can be used as a parameter of the activity of PDNS. The assay, described earlier [21], was modified by us [15].

Incorporation of [8-^{14}C] *hypoxanthine and* [8-^{14}C] *6-mercaptopurine.* At various intervals, cells were incubated with labeled Hx in a final concentration of 10 μM for 20 min at 37°. The amount of intracellular incorporated Hx was assayed as described previously by us [15]. The incorporation of labeled Hx in untreated cells can be used as a parameter of the activity of the purine salvage pathway.

Similar experiments were performed at various intervals with [8-^{14}C] 6MP in a final concentration of 10 μM.

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.* [22] and the levels were expressed as pmol/10^6 viable cells.

RESULTS

Effects of MTX on PRPP levels

Before performing the extensive experiments in the three cell lines, to be described below, we measured PRPP levels in each cell line separately at relatively short periods of time after addition of 0.02 μM and 0.2 μM MTX, respectively, In our further experiments, we measured at 0, 24 and 48 hr after addition of MTX and at some points close to the peak level of PRPP.

Figure 1a shows the effects on intracellular PRPP levels in the three cell lines after incubation with

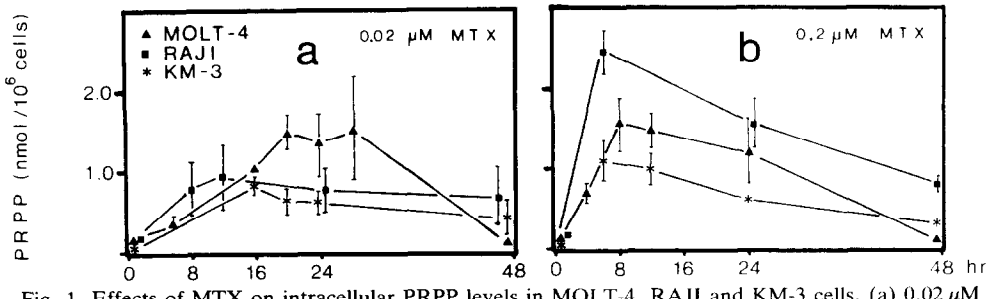

Fig. 1. Effects of MTX on intracellular PRPP levels in MOLT-4, RAJI and KM-3 cells. (a) 0.02 μM MTX, (b) 0.2 μM MTX. Mean ± SD of 3 experiments in duplicate and expressed as nmol/10^6 viable cells. SD not shown when <10% of the levels in untreated cells. PRPP levels in untreated cells were: MOLT-4, 0.125 ± 0.021; RAJI, 0.170 ± 0.018; and KM-3, 0.080 ± 0.007.

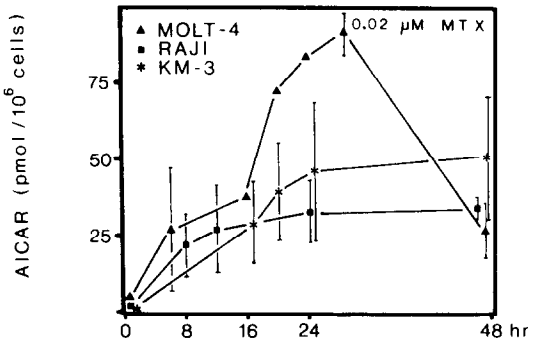

Fig. 2. Effects of 0.02 μM MTX on intracellular AICAR levels in MOLT-4, RAJI and KM-3 cells. Expressed as pmol/10^6 viable cells. See further legend for Fig. 1.

0.02 μM MTX. PRPP levels increased to a maximum after 20–28 hr of incubation in MOLT-4 cells (12-fold increase). The maximum in RAJI cells was reached after 12 hr (6-fold increase). In KM-3 cells a maximum increase of 10-fold was noticed after 16 hr. After 48 hr PRPP levels returned to those of untreated cells in the case of MOLT-4 cells, whereas both in RAJI and KM-3 cells a 5-fold increase persisted. Incubation with 0.2 μM MTX (Fig. 1b) resulted in a 13-fold maximum increase of PRPP levels after 8 hr in MOLT-4 cells, in a 15-fold increase after 6 hr in RAJI cells and in a 14-fold rise after 6 hr in KM-3 cells. At 48 hr PRPP levels in RAJI and KM-3 cells remained increased, whereas those in MOLT-4 cells returned to the levels 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 relatively rapid increase of AICAR was observed with a maximum at 28 hr: after that time levels decreased (Fig. 2). AICAR levels in RAJI and KM-3 cells slowly increased until 24 hr of incubation and then remained constant.

The data on glycine incorporation into purine metabolites (Table 1) after 24 hr of incubation with 0.02 μM MTX indicated a slight, but not significant inhibition of PDNS in MOLT-4 cells, which recovered after 48 hr. Glycine incorporation into purine metabolites in RAJI cells after treatment with 0.02 μM MTX revealed an incomplete, but in time increasing inhibition of PDNS, whereas KM-3 cells demonstrated a complete inhibition of PDNS. It should be noticed that the incorporation of glycine in untreated RAJI cells after 48 hr is lower than after 24 hr. This may be caused by a depletion of an essential cofactor for PDNS in these cells [23].

Incubations with 0.2 μM MTX did not show any increase of AICAR in comparison with untreated cells in all cell lines. With this concentration of MTX almost no glycine incorporation into purine metabolites occurred (Table 1). Both findings indicated a complete inhibition of PDNS by 0.2 μM MTX.

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

Figures 3a and 3b represent the incorporation for 20 min of 10 μM Hx after various periods of incubation with 0.02 μM and 0.2 μM MTX, respectively. The time- and dose-dependent effects were parallel to the intracellular PRPP levels (Fig. 1).

The results of incorporation for 20 min with [8-^{14}C] 6MP in a final concentration of 10 μM after various periods of incubation with MTX are depicted in Figs 4a and 4b. Again, the time- and dose-dependent effects were comparable to Figs 1 and 3. Thus, the time- and dose-dependent increased availability of PRPP after incubation with MTX can be used for enhanced intracellular anabolism by HGPRT into nucleotides [15] of Hx and its analogue, 6MP.

DISCUSSION

MTX and 6MP are the most applied drugs in the maintenance treatment of non-B-non-T- and T-ALL in children [12]. Both antimetabolites inhibit purine *de novo* synthesis: 6MP inhibits a rate limiting step in PDNS: PRPP amidotransferase (EC 2.4.2.14), and MTX inhibits both formyltransferases in PDNS: glycinamide ribonucleotide (GAR) formyltransferase (EC 2.1.2.2) and AICAR formyltransferase (EC 2.1.2.3.). The role of PDNS in the mitogenic response of lymphocytes and in thymocyte dif-

Table 1. Activity of purine *de novo* synthesis in lymphoblasts of different lineages exposed to MTX*

MTX (μM)	MOLT-4	RAJI	KM-3
At 24 hr:			
0	214 ± 38 (100)	139 ± 58 (100)	183 ± 28 (100)
0.02	189 ± 100 (88)	40 ± 29 (29)	2.1 ± 2.1 (1)
0.2	0 (0)	1.8 ± 2.9 (1)	0 (0)
At 48 hr:			
0	231 ± 137 (100)	52 ± 29 (100)	—
0.02	219 ± 71 (94)	10 ± 8 (19)	—
0.2	0 (0)	—	—

* Expressed as pmol incorporated [U-^{14}C] glycine/hr/10^6 viable cells ± SD. The results are the mean of at least 4 experiments. — Not done. Numbers in parentheses: percentages of treated/untreated cells.

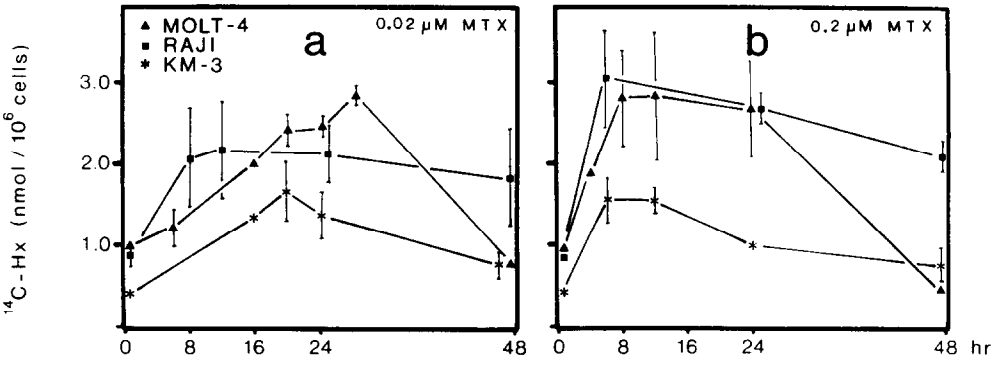

Fig. 3. Effects of MTX on intracellular incorporation of 10 μM [8-^{14}C] hypoxanthine in MOLT-4, RAJI and KM-3 cells: (a) 0.02 μM MTX, (b) 0.2 μM MTX. Expressed as nmol/10^6 viable cells/20 min. See further legend for Fig. 1. Incorporation of 10 μM hypoxanthine in untreated cells: MOLT-4, 0.990 ± 0.110; RAJI, 0.860 ± 0.140; KM-3, 0.420 ± 0.057.

ferentiation has been mentioned in the literature [24–28], and both MTX and 6MP suppress mitogenic responses. Variations in PRPP synthetase (EC 2.7.6.11), in PRPP amidotransferase activity and in overall PDNS activity and availability of PRPP have been reported between normal and stimulated lymphocytes, bone marrow cells and leukemic cells [25, 26, 29–34]. However, few data concerning the activity of PDNS in different subclasses of lymphocytes and malignant lymphoblasts have been reported. We previously demonstrated the importance of an inhibited PDNS with regard to the cytotoxicity of MTX in MOLT-4 malignant T-lymphoblasts [35]. Moreover, we found an enhanced intracellular uptake of 6MP, when 6MP was added to MOLT-4 cells at the moment of maximal inhibition of PDNS by MTX, indicating a potential synergism of the combination of both agents [15]. Differences between three subclasses of malignant lymphoblasts (T-, B- and common ALL-cells) concerning these phenomena are presented in this paper.

The incorporation of labeled Hx in untreated cells (see legend to Fig. 3) can be used as a measure of the activity of the purine salvage pathway [36]. Our data indicate differences in the activity of the purine salvage pathway of the three cell lines. The different activities of the PDNS in untreated lymphoblasts are demonstrated in Table 1. In summary, MOLT-4 cells show a high activity of both pathways, RAJI cells show a rather low PDNS activity and an active salvage pathway, whereas both pathways are moderately active in KM-3 cells.

Figures 1a and 2 and Table 1 demonstrate the events in PDNS due to treatment with 0.02 μM MTX. In RAJI cells, with a low PDNS activity, PRPP levels increase to a maximum at an earlier point of time compared to MOLT-4 cells. PRPP levels remain elevated at 48 hr in RAJI and KM-3 cells, but recover in MOLT-4 cells (Fig. 1a). This is in agreement with almost no inhibition of glycine incorporation in MOLT-4 cells (Table 1), an in time increasing inhibition of PDNS in RAJI cells, and an almost complete inhibition of PDNS in KM-3 cells. The rise of AICAR levels (Fig. 2) indicates a more significant inhibition of AICAR-formyltransferase than GAR-formyltransferase in all cell lines. However, the recovery of AICAR and PRPP levels in MOLT-4 cells indicates a recovery of PDNS, whereas the elevation of PRPP and AICAR levels in RAJI and KM-3 cells indicates a continuation of the inhibition of PDNS in these cell lines. These data suggest that the critical concentration of 0.02 μM MTX is able to produce MTX polyglutamates in RAJI and KM-3 cells, but not in MOLT-4 cells. MTX polyglutamates are more potent and direct inhibitors of AICAR formyltransferase compared to MTX, whereas the effects on GAR formyltransferase are less impressive [37, 38]. Thus, the net result is a much more pro-

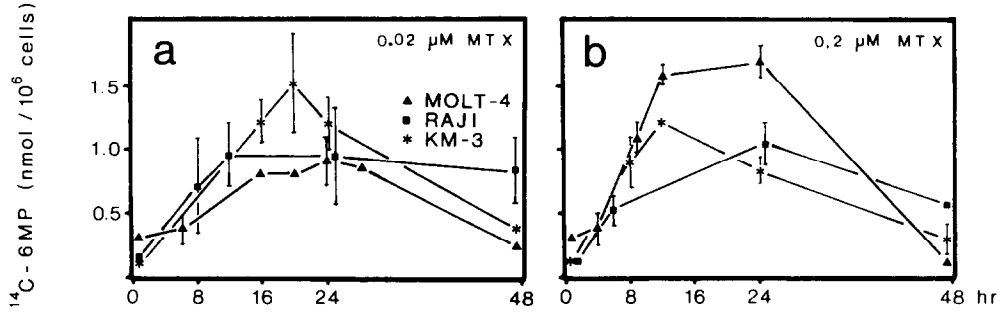

Fig. 4. Effects of MTX on intracellular incorporation of 10 μM [8-^{14}C] 6-mercaptopurine in MOLT-4, RAJI, and KM-3 cells. (a) 0.02 μM MTX, (b) 0.2 μM MTX. Expressed as nmol/10^6 viable cells/20 min. See further legend for Fig. 1.

nounced and direct inhibition of AICAR formyltransferase in RAJI and KM-3 cells associated with a continuous increase of AICAR levels. The changes of dTTP levels after treatment with 0.02 μM MTX are also in line with formation of MTX polyglutamates in RAJI and KM-3 cells and not in MOLT-4 cells [39].

The results of incubation with 0.2 μM MTX with respect to the PDNS of MOLT-4, RAJI and KM-3 cells were almost identical: glycine incorporation was completely inhibited in all cell lines and AICAR levels were not detectable, indicating a complete inhibition of both formyltransferases in PDNS. PRPP levels (Fig. 1b) raised to the same extent after similar periods of time. However, the maximal increase of PRPP levels in all cell lines occurred earlier than in cells treated with 0.02 μM MTX. After 48 hr of incubation with 0.2 μM MTX, PRPP levels in MOLT-4 cells returned to those of untreated cells, whereas PRPP levels in RAJI and KM-3 cells decreased less and remained elevated 5-fold and 4-fold, respectively. Table 1 indicates that this cannot be attributed to a relief of the inhibition of PDNS, as was the case in MOLT-4 cells treated with 0.02 μM MTX. Earlier studies suggested that the decrease of PRPP synthesis was attributed to a decreased availability of the co-substrates ATP and ribose-5-phosphate [23, 27, 40–43]. However, we could demonstrate unchanged or even increased levels of ATP 48 hr after treatment with MTX [35, 39]. Therefore, the lowering of PRPP levels after 48 hr have to be attributed to a greater cytotoxic sensitivity of MOLT-4 cells compared to RAJI and KM-3 cells, associated with nucleic acid breakdown and re-utilization of purine and pyrimidine precursors, consuming PRPP as a cofactor [44–48].

The increased availability of PRPP due to inhibition of PDNS after treatment with MTX can be used for an enhanced incorporation of hypoxanthine (Fig. 3). The amount of incorporated hypoxanthine at each point of time and within each cell line is directly correlated with the PRPP levels. These time- and dose-dependent effects indicate that administration of natural purine bases and thymidine is able to rescue a prolonged purineless and thymidylateless state after treatment with MTX [40, 41, 45–67].

It should be emphasized, that the *in vitro* situation in our investigations may be different from the *in vivo* situation. The influence of variations in Hx levels in the medium on the activity of PDNS and the synergistic effect of MTX and purine or pyrimidine analogues was subject of many studies [40, 63, 68–72]. We could demonstrate a decrease of the initial concentration of Hx in the culture medium from 3–5 μM to 0.1–0.2 μM after 24 and 48 hr due to consumption by growing cells via the salvage pathway, irrespective of the presence of MTX. Concentrations of 3–5 μM Hx in the medium were able to inhibit PDNS [36, 43, 69], but did not modulate cytotoxicity [65]. Concentrations of 0.1–0.2 μM were unable to inhibit PDNS. Thus, the Hx exhaustion in our studies is of minor importance with respect to the effects of MTX.

Figure 4 demonstrates that the increased availability of PRPP can also be used for an enhanced incorporation of the Hx analogue, 6MP. Similar time- and dose-dependent effects were obtained as in the case of PRPP and Hx (Figs 1 and 3). The absolute amount of 6MP incorporated in untreated cells is approximately 2–5 times lower than the amount of Hx incorporated. This can be explained by the fact that 6MP can only be incorporated into nucleic acids as thioguanine (deoxy)ribonucleotides, whereas Hx is incorporated into nucleic acids in the form of both adenine and guanine (deoxy)ribonucleotides [25, 73]. The absolute amount of incorporation of 6MP in untreated cells is correlated with the activity of the purine salvage pathway. The time period of increased incorporation in MOLT-4 cells with an active PDNS is shorter: after 48 hr of incubation with MTX the incorporation of Hx and 6MP in MOLT-4 cells almost returned to levels of untreated cells, whereas it was still increased in RAJI and KM-3 cells. Moreover, it is a conspicuous phenomenon that the ratio of incorporation of 6MP in treated cells/untreated cells at each point of time is higher in RAJI and KM-3 cells than the ratio of Hx incorporation. This is not the case in MOLT-4 cells. These data indicate that 6MP is more actively incorporated than Hx in RAJI and KM-3 cells in the presence of increased PRPP levels due to pretreatment with MTX. These observations suggest that the inhibition of PRPP amidotransferase by methylthio-IMP, after incubation with 6MP, is more pronounced in RAJI and KM-3 cells. The increased inhibition of PDNS by the combination of MTX and 6MP results in a further increase of PRPP levels, which can be used for a further increase of 6MP incorporation (self-enhancement). With respect to Hx this self-enhancement is absent.

In conclusion, our data suggest that RAJI and KM-3 cells are more vulnerable for combination treatment of MTX and 6MP than MOLT-4 cells. Cytotoxicity studies, using various combinations of MTX and 6MP in these cell lines, are in agreement with these metabolic findings (manuscript in preparation).

The results of these *in vitro* investigations have to be translated to the *in vivo* situation. Because both Hx and 6MP are competitive substrates for HGPRT, the plasma concentrations of 6MP *in vivo* should be equivalent or higher than the plasma concentrations of Hx, especially in bone marrow. We found high Hx values in bone marrow of patients after cessation of maintenance therapy for leukemia: 6.9 ± 6.4 μM (N = 154), whereas the ratio bone marrow Hx/plasma Hx was 10.6 (N = 81, range 1.1–56). These are in concordance with bone marrow Hx concentrations found by others [60, 74, 75]. In order to obtain equivalent 6MP concentrations in bone marrow *in vivo*, intravenous or high dose oral 6MP administration will be necessary [76]. Therefore, further studies in children with T-ALL, B-ALL and cALL are necessary in order to define the optimal sequence, dose and route of administration of MTX and 6MP for a maximum benefit from the synergism of both drugs.

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