Biochemical Pharmacology, Vol. 45, No. 7, pp. 1455–1463, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

6-MERCAPTOPURINE: CYTOTOXICITY AND BIOCHEMICAL PHARMACOLOGY IN HUMAN MALIGNANT T-LYMPHOBLASTS

JOS P. M. BÖKKERINK,* ELISABET H. STET, RONNEY A. DE ABREU, FRANK J. M. DAMEN, TILLY W. HULSCHER, MARINKA A. H. BAKKER and JOHN A. VAN BAAL

Center for Pediatric Oncology S.E. Netherlands, Department of Pediatrics, St. Radboud University Hospital of Nijmegen, 6500 HB Nijmegen, The Netherlands

(*Received* 9 *April* 1992; *accepted* 18 *December* 1992)

Abstract—The effects of prolonged exposure to 2 and 10 μM 6-mercaptopurine (6MP) in the human lymphoblastic T-cell line MOLT-4 were studied with respect to cell-kinetic parameters, phosphoribosyl pyrophosphate (PRPP) and purine ribonucleotide levels, formation of 6MP-nucleotides, especially methyl-thio-IMP (Me-tIMP), DNA and RNA synthesis ([^{32}P] incorporation), and [8-^{14}C]6MP incorporation into newly synthesized DNA and RNA. The results provided new insights into the complex mechanism of action of 6MP in human malignant lymphoblasts. Exposure to 2 μM 6MP resulted in a rapid inhibition of purine *de novo* synthesis (PDNS) by increased levels of Me-tIMP, resulting in increased PRPP levels and decreased purine ribonucleotides, affecting cell growth and clonal growth, and less cell death. DNA synthesis decreased, associated with an increasing delay of cells in S phase. Incorporation of thioguanine nucleotides into newly synthesized DNA resulted in an increasing arrest of cells in G_2 + M phase. RNA synthesis, initially decreased, recovered partially, associated with a recovery of purine ribonucleotides. New formation of 6MP-nucleotides (tIMP) was only detected within the first 24 hr, and 6MP levels in the culture medium were already undetectable after 6 hr of exposure to 2 μM, indicating a high rate of incorporation and complete conversion of 6MP within this period. Incorporation of 6MP-nucleotides into DNA was 5 times as high as incorporation into RNA. Exposure to 10 μM 6MP resulted in early cytotoxicity at 24 hr, associated with a complete inhibition of PDNS by a large pool of Me-tIMP and lower levels of purine ribonucleotides as compared to 2 μM 6MP. A more severe delay of cells in S phase was associated with an inhibition of DNA synthesis to 14% of control within the first 24 hr, and an arrest in G_2 + M phase. Further increasing levels of Me-tIMP caused an arrest of cells and late cytotoxicity in S phase at 48 hr, preventing further progression into G_2 + M phase. Our data suggest that inhibition of PDNS due to Me-tIMP is a crucial event in the mechanism of 6MP cytotoxicity. It is responsible for decreased RNA synthesis and decreased availability of natural deoxyribonucleotides, causing a delay of DNA synthesis in S phase. This enhances incorporation of 6MP as thioguanine nucleotides into DNA in the S phase and subsequent late cytotoxicity in the G_2 phase. However, with high concentrations of 6MP, the large pool of Me-tIMP causes severe reduction of natural deoxyribonucleotides in lymphoblasts with an active PDNS. This is responsible for pronounced inhibition of DNA synthesis and early cytotoxicity in the S phase.

6-Mercaptopurine (6MP†) has been used as an important agent in the maintenance treatment of childhood non-B acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma since its introduction in 1952 [1] and the first clinical studies in 1953 [2]. Although many studies focussed on the anabolism and catabolism of 6MP and on the biochemical effects of 6MP-metabolites on purine metabolism, many questions regarding the exact mechanism of 6MP cytotoxicity remained obscure or have been subject to debate in the literature. The role of the 6MP-metabolites, 6-thioIMP (tIMP) and 6-methyl-thioIMP (Me-tIMP), with regard to the allosteric inhibition of the first enzyme in purine *de novo* synthesis (PDNS), phosphoribosyl pyrophosphate (PRPP) amidotransferase (EC 2.4.2.14), has been studied extensively and Me-tIMP proved to be the more effective inhibitor of PDNS [3–9]. The non-competitive inhibition of adenylosuccinate synthetase (EC 6.3.4.4), the competitive inhibition of adenylosuccinate lyase (EC 4.3.2.2), and the inhibition of IMP dehydrogenase (EC 1.2.1.14) in the purine interconversion pathway [5, 7, 10] requires relatively high concentrations of 6MP(-metabolites) and might not have clinical importance. The contribution of the inhibition of PDNS to early cell growth inhibition or early cytotoxicity of 6MP has been discussed extensively in the literature [8, 11–15]. However, the most important mechanism of cytotoxicity of 6MP was shown to be incorporation as 6-thioguanine (6TG) deoxyribonucleotides into DNA [13, 14, 16].

Although human malignant lymphoblasts were the most susceptible cells to exposure of 6MP among

* Corresponding author. Tel. (080) 516928; FAX (080) 540576.

† Abbreviations: ALL, acute lymphoblastic leukemia; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; (Me-)tIMP, (methyl-)thio-IMP; MTX, methotrexate; PDNS, purine *de novo* synthesis; PRPP, phosphoribosyl pyrophosphate; TPMT, thiopurine methyltransferase; 6MP, 6-mercaptopurine; 6TG, 6-thioguanine; DTT, dithiothreitol; DAPI, 4′,6-diamino-2-phenylindole.

human malignancies [2, 11–13, 17–21], studies with respect to all aspects of its mechanism of action in human lymphoblastic cells are scarcely available.

This study presents the effects of prolonged exposure to 2 and 10 μM 6MP on cell-kinetic parameters (cell growth, cell viability, clonal growth, flow cytometry), on Me-tIMP levels, on PDNS (PRPP levels) and purine ribonucleotides, and double labeling experiments, concerning incorporation of [8-^{14}C]6MP into newly synthesized DNA and RNA, measured by incorporation of [^{32}P]phosphate.

MATERIALS AND METHODS

[8-^{14}C]6MP (1.7 mCi/mmol) and [^{32}P]phosphoric acid were purchased from Amersham International (Amersham, U.K.); PRPP and calf thymus DNA from the Sigma Chemical Co. (St Louis, MO, U.S.A.); a preparation from brewer's yeast containing orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) and orotidylate decarboxylase (ODC, EC 4.1.1.23), Pronase, 4',6-diamino-2-phenylindole (DAPI), and dithiothreitol (DTT) from Boehringer Mannheim (Mannheim, F.R.G.); Aqua Luma scintillation fluid from Lumac 3M (Schaesberg, The Netherlands).

The cell line MOLT-4 (T-lymphoblasts) has been maintained in continuous culture in our laboratory [22–25]. The absence of mycoplasma contamination, and the presence of antigens on the cells in culture was tested regularly.

Logarithmically growing cells (doubling time 24 hr) were suspended in fresh RPMI 1640 Dutch Modification medium in a concentration of 0.3×10^6 cells/mL 24 hr before each experiment. The conditions for cell culture, soft agar colony forming activity, flow cytometry, and the PRPP assay were identical to those described earlier by us [24, 25]. Cell number and viability (trypan blue exclusion) were determined in duplicate in a Bürker–Türk chamber. Cell growth was expressed as percentage of viable untreated cells. The number of non-viable cells was corrected for non-viable untreated cells (±5%) at each time point. Colony-forming activity was expressed as percentage of plating efficiency of untreated cells at each time point (mean plating efficiency: 7%), and the results were plotted on a semi-logarithmical scale. [8-^{14}C]-6MP, from a stock solution containing 60 mg/mL DTT in order to prevent desulfuration of 6MP [26], and [^{32}P]phosphoric acid were diluted in a small volume of RPMI 1640 medium (0.002–0.01 fraction), and were added as a single dose at time points indicated below.

The HPLC assay of normal intracellular ribonucleotides was described by us earlier [25]. Me-tIMP was detected by the same assay at a wavelength of 290 nm.

Incorporation of [8-^{14}C]*6MP and/or* [^{32}P]*phosphate into newly synthesized DNA and RNA.* Final concentrations of 2 and 10 μM [8-^{14}C]6MP, respectively, were added to 11 mL of the cell suspension after 24 hr of preincubation. All experiments were performed in duplicate. In order to measure the amount of newly synthesized DNA and RNA during the first and the second 24 hr after addition of 6MP or in untreated cells, the cell suspension was exposed to [^{32}P]phosphate (further indicated as [^{32}P]) in a final concentration of 1 μCi/mL at 0 or 24 hr. After 24 hr incubation with [^{32}P] in the presence or absence of [8-^{14}C]6MP, the cell suspension was divided into five vials, each containing 2 mL of the cell suspension; four for quadruplicate determinations of [8-^{14}C] and/or [^{32}P] incorporation. In the fifth sample the DNA content of the cell suspension was measured after the indicated incubation period, using a fluorometric DNA assay with DAPI [27]. Furthermore, 100 μL of the cell suspension in liquid scintillation fluid was counted in duplicate in a liquid scintillation analyser for determination of the specific activity of [^{32}P] at the time of measurement of the samples. The vials were centrifuged (9500 *g*; 3 min) and the cell pellets were washed three times with 0.9% NaCl solution and stored at −20°. In order to remove all protein, to destroy the cell membranes, and to precipitate DNA and RNA, we used the method described by Kufe *et al.* [28]. The cell pellet was resuspended in 0.5 mL of 0.01 M Tris buffer (pH 7.4), containing 0.1 M EDTA, 0.5% sodium dodecyl sulfate and freshly dissolved nuclease-free Pronase (2 mg/mL). After 30 sec careful sonification incubations were performed for 3 hr at 37°. In order to precipitate both DNA and RNA, the samples were frozen after mixing with 100 μL 4 M NaCl and 1.2 mL ethanol 100% for 24 hr at −20°. After centrifugation (8500 *g*; 5 min) the supernatants, containing 6MP-nucleotides, were evaporated at 60°, resuspended in 1 mL water and 15 mL scintillation fluid, and radioactivity was determined. The pellets, containing DNA and RNA, were washed twice at 0° with 100% ethanol and evaporated at 60°. In order to measure the incorporation of [8-^{14}C]6MP and/or [^{32}P] into RNA, the RNA was hydrolysed during incubation for 1 hr at 37° with 0.4 M KOH solution, containing also 0.5 mg/mL calf thymus DNA. Calf thymus DNA was used as a carrier during precipitation of DNA. After cooling on ice DNA was precipitated for 1 hr by addition of 100 μL 4 M $HClO_4$. After brief centrifugation the supernatant, containing RNA, was neutralized to pH 7.0 with 0.5 mL of a solution, containing 0.4 M KOH and 0.1 M K_2HPO_4, and radioactivity was determined after addition of scintillation fluid. The pellet containing DNA was resuspended in 1 mL of a solution, containing 0.04 M KOH and 0.01 M K_2HPO_4, and radioactivity was counted after addition of 15 mL scintillation fluid.

Final results of incorporation of [8-^{14}C]6MP and/or [^{32}P] into DNA and RNA and the formation of [8-^{14}C]6MP-nucleotides were expressed as nmol/mg DNA after correction for the specific activity of [^{32}P] and the overlap of [^{32}P] counts in the [^{14}C] channel.

RESULTS

Effects of 6MP on cell kinetic parameters and on changes in DNA distribution

The effects of 2 and 10 μM 6MP, respectively, on cell growth, cell viability and colony-forming activity are shown in Fig. 1A–C. A time- and concentration-dependent effect of 6MP can be noted on all parameters. A sign test with respect to the average percentages (averages over 24, 48 and 72 hr) gave a

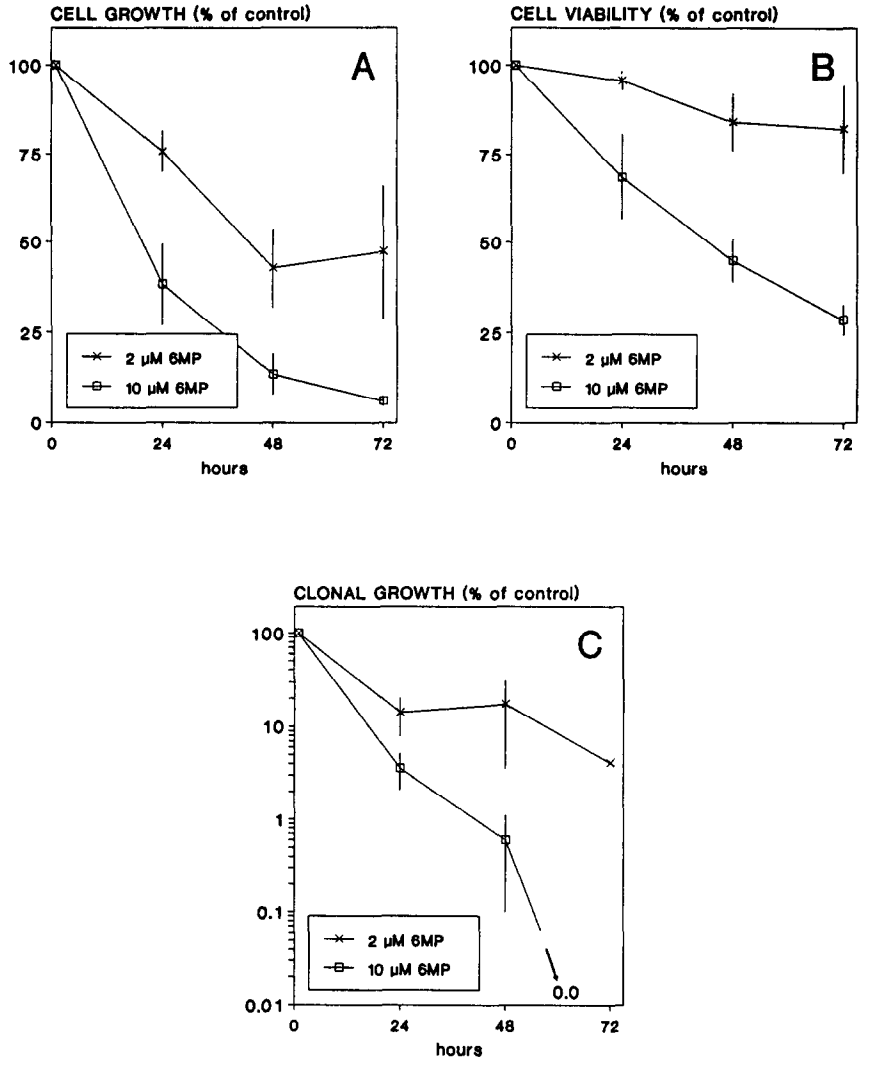

Fig. 1. Cell growth characteristics of MOLT-4 cells after exposure to 2 and 10 μM 6MP. (A) Cell growth; (B) cell viability; (C) clonal growth. Expressed as percentages of untreated cells at each time point; mean ± SD of 14 experiments in duplicate; clonal growth: 5 experiments in duplicate, except for 72 hr: 1 experiment.

significantly lower level for cell growth and cell viability ($P = 0.001$) and a nearly significantly lower level for clonal growth (excluding 72 hr, $P = 0.06$) after exposure with 10 μM 6MP. Cell viability and clonal growth are less affected after prolonged incubation with 2 μM 6MP and indicate incomplete cytotoxicity and incomplete inhibition of clonal growth capacity, and some recovery at 72 hr. The effects of prolonged incubation with 10 μM 6MP are complete. The effects of 2 μM 6MP on changes in DNA distribution (Fig. 2A) demonstrate an increasing delay of cell progression in S phase and an arrest in G_2 + M phase, associated with a decrease of cells in G_1 phase. The effects of 10 μM 6MP on cell progression in the first 24 hr are similar, but more severe (Fig. 2B). In contrast to 2 μM 6MP, the amount of cells in G_2 + M phase decreases at 48 hr with 10 μM 6MP, indicating a complete arrest of cell progression in S phase.

Effects of 6MP conversion into PRPP and normal purine ribonucleotide levels

Exposure to both concentrations of 6MP resulted in a rapid increase of intracellular PRPP levels (Fig. 3) with a maximum after 6 hr. Pairwise comparison according to Nemenyi [29] with respect to the average PRPP levels from 1.5–12 hr for 2 and 10 μM 6MP and untreated cells only gave a significantly higher level for 2 μM with respect to untreated cells. A similar comparison from 31.5–48 hr only demonstrated a significantly higher level for 2 μM with respect to 10 μM ($P = 0.02$). PRPP levels remained elevated after exposure to 2 μM 6MP, whereas a rapid decrease was noted after 6-hr

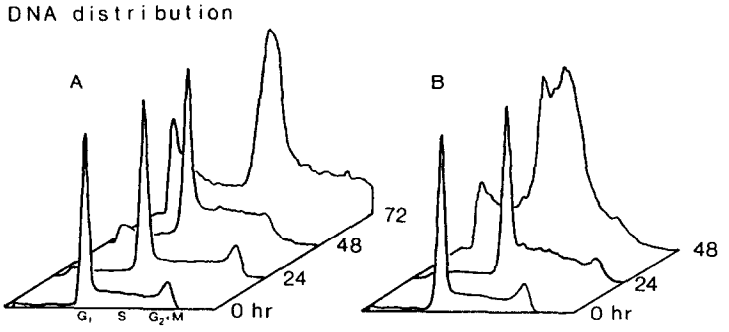

Fig. 2. Effects of 2 and 10 μM 6MP on DNA distribution of MOLT-4 cells, measured by flow cytometry. In percentages; mean of 3 experiments.

	(A) 2 μM 6MP			(B) 10 μM 6MP		
	G_1	S	$G_2 + M$	G_1	S	$G_2 + M$
0 hr	54.3	37.0	8.7	54.3	37.0	8.7
24 hr	51.6	38.3	9.9	48.4	41.4	10.2
48 hr	42.2	47.2	10.6	38.7	54.2	7.0

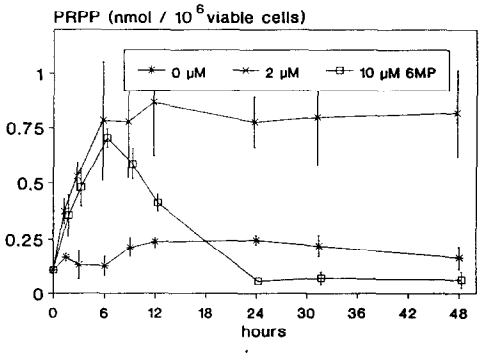

Fig. 3. Intracellular PRPP levels in MOLT-4 cells after exposure to 2 and 10 μM 6MP. Expressed as nmol/10^6 viable cells; mean ± SD of 4 experiments in duplicate.

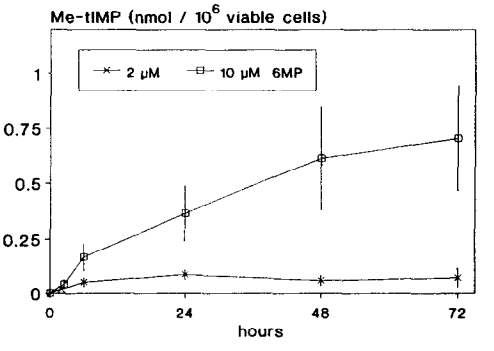

Fig. 4. Intracellular Me-tIMP levels in MOLT-4 cells after exposure to 2 and 10 μM 6MP. Expressed as nmol/10^6 viable cells; mean ± SD of 4 experiments in duplicate.

exposure to 10 μM 6MP below levels of untreated cells. Intracellular Me-tIMP (Fig. 4) levels rapidly increased after 2 hr with a maximum at 24 hr after exposure to 2 μM 6MP followed by a slow decrease. 6MP (10 μM) resulted in a continuous increase of Me-tIMP levels. However, because of the small number (four) of experiments with complete observations, the sign test did not give a significant difference between both concentrations with respect to the average level ($P = 0.13$).

The inhibition of PDNS by 2 μM 6MP was accompanied by an initial decrease of ATP and GTP levels (Fig. 5A). This was followed by a recovery of ATP and GTP pools between 24 and 48 hr. After exposure to 10 μM 6MP (Fig. 5B) purine ribonucleotides showed a more severe depletion, due to complete inhibition of PDNS. After 6–24 hr ATP and GTP levels recovered partially.

Incorporation of [8-^{14}C]*6MP and/or* [^{32}P]*phosphate into newly synthesized DNA and RNA*

The extensive double labeling experiments were performed to gain insight into the effects of 6MP on DNA and RNA synthesis and the formation of 6MP-nucleotides. The effects on DNA and RNA synthesis are demonstrated in Fig. 6. Exposure to 2 μM 6MP lowered the phosphate incorporation into DNA and RNA within the first 24 hr to 35% and 26%, respectively, of control cells, whereas 10 μM 6MP lowered DNA and RNA synthesis to 14% and 16%, respectively. A two-way analysis of variance (mixed model by considering the separate experiments as levels of a random factor) did not give a significant difference between both concentrations ($P = 0.12$) with respect to both DNA and RNA. In the second 24 hr after exposure to 2 μM 6MP DNA and RNA

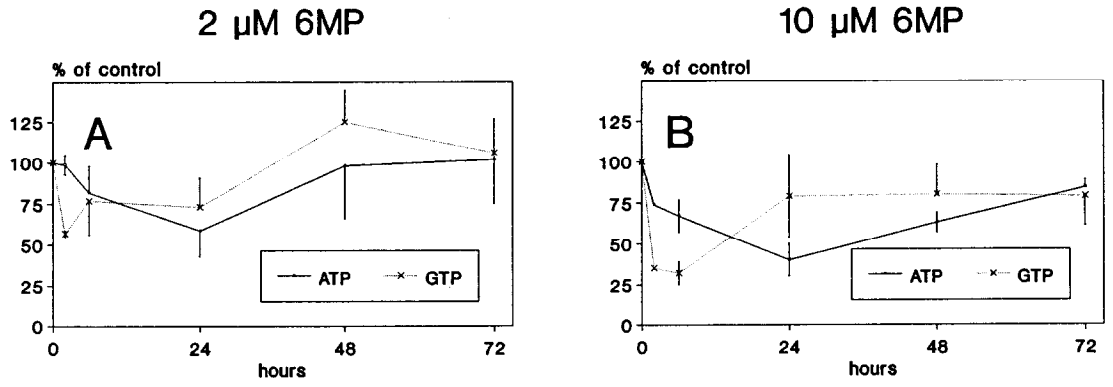

Fig. 5. Intracellular ATP and GTP levels after exposure to (A) 2 μM and (B) 10 μM 6MP. Expressed as percentages of untreated cells; mean ± SD of 5 experiments in duplicate. Untreated viable cells: ATP 4746 ± 637 pmol/10^6 cells and GTP 891 ± 116 pmol/10^6 cells (N = 11).

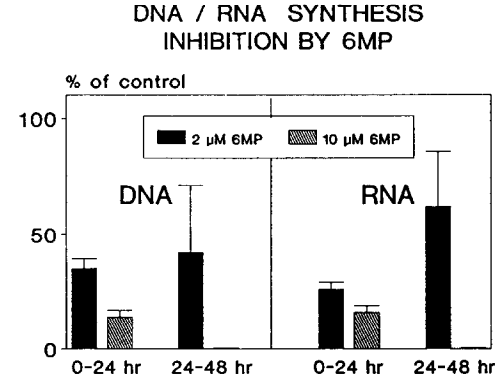

Fig. 6. DNA and RNA synthesis in MOLT-4 cells, measured by [^{32}P]phosphoric acid incorporation, after exposure to 2 and 10 μM 6MP. Expressed as percentages of untreated cells; mean ± SD of quadruplicate determinations in 2 experiments in duplicate.

synthesis increased to 42% and 62%, respectively. Incorporation studies during the second 24 hr with 10 μM 6MP were not performed due to its excessive cytotoxic effects in this time period (Fig. 1).

A clear difference between RNA synthesis and DNA synthesis within the first 24 hr could not be established. Separate signed rank tests for the four dosis-experiment combinations gave conflicting results (lower RNA levels for dosis 2 μM with P = 0.008 and P = 0.08 and higher levels for RNA levels for dosis 10 μM with P = 0.02 and P = 0.22). The comparison DNA versus RNA synthesis from 24–48 hr gave a significantly higher RNA level (signed rank test: P = 0.008).

The incorporation of [8-^{14}C]6MP into DNA, RNA and nucleotides is shown in Table 1. The ratio of incorporation of 2 μM 6MP into DNA, RNA and nucleotides in the first 24 hr is 4.5:1:9, and in the second 24 hr 5.5:1:0, indicating that 6MP is incorporated far more into DNA in comparison to RNA (sign tests: P ≤ 0.02 and P = 0.02, respectively). We were unable to measure formation of 6MP-nucleotides in the second 24 hr after exposure to 2 μM 6MP. These data are in agreement with the HPLC-measurements of 6MP levels [30] in the culture medium after exposure to 2 μM 6MP, shown in Table 2. After 6 hr of incubation with 2 μM 6MP we could not detect a measurable amount of 6MP in the culture medium, indicating a very rapid uptake and complete conversion of 2 μM 6MP.

In cells treated with 10 μM 6MP, the ratio of 6MP incorporation into DNA, RNA and nucleotides in the first 24 hr is 3.9:1:12.9 (Table 1), also indicating more incorporation of 6MP into DNA as compared to RNA (sign tests: P = 0.02). However, the majority of 6MP is incorporated into nucleotides. This is in agreement with the continuous increase of Me-tIMP levels (Fig. 4). Table 2 indicates that approximately 70–75% of the total available amount of 10 μM 6MP is incorporated into the cells, suggesting a maximum plateau of intracellular uptake.

In order to account for the inhibition of DNA and RNA synthesis by 6MP and its cytotoxic effects, the incorporation of [8-^{14}C] into RNA and DNA was expressed as percentage of [^{32}P] incorporation into these compounds (Fig. 7). In this way we could determine to what extent 6MP-nucleotides are incorporated into newly synthesized DNA and RNA in comparison to natural nucleotides. These data demonstrate that the amount of 6MP incorporation into DNA after exposure to 2 μM 6MP is 3.6% of total nucleotide incorporation into DNA in both time periods. The incorporation into RNA is more pronounced in the first 24 hr, as compared to the second 24 hr. Moreover, Fig. 7 demonstrates that 6MP preferentially incorporates into DNA in comparison to RNA (sign tests: P ≤ 0.02). These findings are more obvious in experiments with 10 μM 6MP: 6MP-nucleotides form approximately 15% of total nucleotide incorporation into DNA, whereas DNA synthesis is severely inhibited to 14% of control (Fig. 6) in the first 24 hr. In contrast, the amount of 6MP incorporated into RNA is only 3.2% of total nucleotide incorporation, whereas RNA synthesis is inhibited to the same extent as DNA synthesis.

Table 1. [8-^{14}C]6-MP incorporation into DNA, RNA and nucleotides of MOLT-4 cells

	DNA		RNA		6MP-nucleotides	
	0–24 hr	24–48 hr	0–24 hr	24–48 hr	0–24 hr	24–48 hr
2 μM 6MP	6.9 ± 0.2	7.7 ± 0.8	1.5 ± 0.1	1.4 ± 0.6	13.8 ± 2.0	0
10 μM 6MP	11.0 ± 2.5	—	2.8 ± 0.6	—	36.0 ± 19.7	—

Expressed as nmol/mg DNA.
Mean ± SD of quadruplicate measurements in 2 experiments in duplicate.
—, not measured.

Table 2. Concentrations of 6MP in the culture medium after exposure to 2 and 10 μM 6MP

Duration of exposure (hr)	0.5	2.5	4	6	8	24	48
[6MP] in culture medium:							
2 μM 6MP	0.8	0.5	0.3	0	0	0	0
10 μM 6MP	4.4	3.0	2.6	3.0	2.4	2.2	2.9

Expressed as μM.
Mean of 2 experiments.

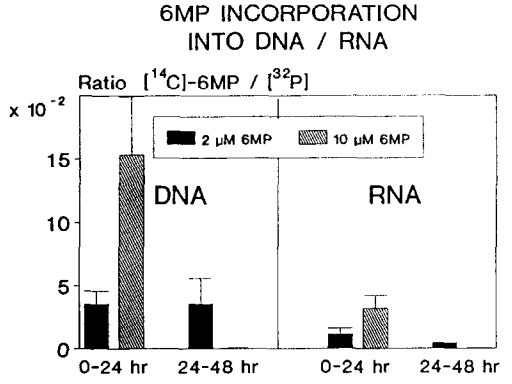

Fig. 7. Ratio [8-^{14}C]6MP/[^{32}P]incorporation into DNA and RNA of MOLT-4 cells after exposure to 2 and 10 μM 6MP (×10^{-2}; mean ± SD of quadruplicate determinations in 2 experiments in duplicate).

DISCUSSION

The effects of 6MP have been studied extensively in various malignant cell lines. Its efficacy was proven in many non-lymphoid or non-human experimental *in vitro* and *in vivo* models. Nevertheless, the agent did not arouse interest in the treatment of various cancers [17–19] and was ineffective in various pediatric round cell tumors [20]. Until now, 6MP is predominantly used in the maintenance treatment of ALL and non-Hodgkin's lymphoma. The combination of oral 6MP and methotrexate (MTX) proved to be superior compared to each agent separately in the early treatment of ALL [31]. In previous studies we demonstrated the biochemical basis for a selective synergism of MTX and 6MP in human malignant lymphoblasts of three immunological subclasses [22, 24]. These investigations formed the background of the present extensive study, concerning the cytotoxicity and biochemical effects of 6MP in MOLT-4 cells, using concentrations which gave rise to sufficient cytotoxicity, which could easily be maintained during prolonged intravenous administration *in vivo*, and which also resulted in cytotoxic cerebrospinal fluid levels [32, 33].

Our data suggest the following sequence of events after treatment with 2 μM 6MP: after a rapid intracellular uptake, all 6MP disappears from the medium within 6 hr (Table 2) and is converted into tIMP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8), consuming PRPP. tIMP and especially Me-tIMP, formed by methylation of tIMP, are known inhibitors of the first enzyme in PDNS, PRPP amidotransferase [3–5]. Me-tIMP levels increased already after 2 hr exposure (Fig. 4). The inhibition of PDNS by Me-tIMP was demonstrated by a rapid rise of intracellular PRPP levels with a maximum after 6 hr (Fig. 3). The inhibition of PDNS resulted in decreased levels of purine ribonucleotides (Fig. 5A) between 2 and 24 hr, causing an inhibition of RNA synthesis to 26% (Fig. 6). The inhibition of PDNS also resulted in a decrease of purine deoxyribonucleotide levels [15], and was demonstrated in our experiments by a delay of cell progression through the S phase (Fig. 2A) [34, 35] and an inhibition of DNA synthesis to 35% (Fig. 6). These biochemical events cause early cell growth and clonal growth inhibition (Fig. 1A and C), but affect cytotoxicity less (Fig. 1B).

Data from the literature indicated that the effects of inhibition of PDNS were reversible, when cells were resuspended in fresh medium [11, 12, 14], associated with a decreased formation of thiopurine nucleotides [12, 21]. These and other studies indicated that late cytotoxicity of 6MP (and its analog thioguanine, 6TG) was due to incorporation as 6TG-

deoxyribonucleotides into DNA during the S phase [13, 14, 16, 36], suggesting that cells in early S phase are the most sensitive to 6MP exposure [37–39]. After progression through the S phase cells which incorporated 6TG-deoxyribonucleotides into DNA are arrested in the G_2 phase of the cell cycle, associated with chromosomal curling and unilateral DNA strand breaks [35, 36, 40, 41]. Figure 2A demonstrates that the percentage of cells in G_2 + M phase already increased after 24 hr, associated with a decrease of cells in G_1 phase, indicating an arrest of a number of cells in G_2 phase already after 24 hr. However, the majority of cells is not in S phase at the onset of exposure to 6MP; these cells incorporate 6MP into DNA in S phase at a later time point and this causes late cytotoxicity of 6MP in the subsequent G_2 phase (between 24 and 48 hr). This was confirmed by a further accumulation of cells in the S and G_2 + M phase at 48 hr (Fig. 2A).

Our data indicate that the inhibition of PDNS by Me-tIMP is a crucial event in the mechanism of 6MP cytotoxicity. The depletion of normal purine nucleotides by the inhibition of PDNS results in an inhibition of DNA synthesis especially in early S phase (Fig. 2A). As false thiopurine nucleotides are abundantly available, an enhanced incorporation of thioguanine nucleotides for the remaining new synthesis of DNA in early S phase will occur. This explains the predominance of 6MP incorporation into DNA, which is almost five times higher as into RNA (Table 1). The predominance of 6TG incorporation into DNA during early S phase, due to the inhibition of PDNS, will ultimately result in enhanced cell death in G_2 phase, demonstrated by an increasing arrest of cells in G_2 + M phase at 48 hr (Fig. 2A).

Figure 6 indicates that RNA synthesis recovered between 24 and 48 hr, due to a recovery of ribonucleotide levels (Fig. 5A). This recovery may be caused by the following phenomena. Although new formation of Me-tIMP was absent after 24 hr, due to absence of 6MP in the medium (Tables 1 and 2), the long half-life of Me-tIMP [42] (Fig. 4) resulted in persistent inhibition of PDNS between 24 and 48 hr, as demonstrated by increased availability of PRPP (Fig. 3). As all 6MP is already converted, PRPP can be used now for reutilization of purine and pyrimidine precursors from nucleic acid breakdown due to increased loss of viable cells, leading to increased ribonucleotide levels and recovery of RNA synthesis in viable cells.

The effects of exposure to 10 μM 6MP demonstrate important differences with 2 μM 6MP. A rapid formation of tIMP was found with a maximum at 6 hr (data not shown), in even higher amounts as reported by Zimm *et al.* [21]. These authors did not measure exactly the formation of Me-tIMP in MOLT-4 cells, because tIMP and Me-tIMP could not accurately be separated in their assay. By indirect measurement Me-tIMP levels were less than 5% of tIMP and Me-tIMP levels together [21]. With our assay, however, Me-tIMP levels were already 30% of tIMP levels at 6 hr after incubation with 10 μM 6MP and increased further (Fig. 4). The initial increase of PRPP levels was similar when compared to 2 μM 6MP (Fig. 3), and the inhibition of PDNS resulted in a severe depletion of purine ribonucleotide levels (Fig. 5B). The subsequent decrease of PRPP levels after 6 hr and the (partial) recovery of ribonucleotide levels seem to be contradictory to a further increase of Me-tIMP levels and a complete cessation of PDNS. Therefore, these phenomena merely point to a maximal consumption of PRPP for the conversion of 6MP by HGPRT, exceeding the synthesis of PRPP. Even the maximal consumption of PRPP was insufficient to incorporate all 6MP from the medium (Table 2). Moreover, increased consumption of PRPP may be caused by reutilization of purine and pyrimidine precursors from nucleic acid breakdown due to severe loss of viable cells (Fig. 1B), resulting in increased levels of ribonucleotides at 48 hr (Fig. 5B), without consumption for and recovery of RNA synthesis, similar to our previous studies with MTX [23, 25].

The severe perturbations of nucleotide levels due to complete inhibition of PDNS are responsible for a more significant delay of cell progression through the S phase as compared to 2 μM 6MP (Fig. 2B). As DNA synthesis is inhibited to 14% (Fig. 6), mainly due to severe delay of cell progression through the S phase, the incorporation of thioguanine nucleotides into newly synthesized DNA in S phase is significantly increased in comparison to 2 μM 6MP (Table 1, Fig. 7). This is accompanied by a more pronounced arrest in G_2 + M phase at 24 hr (Fig. 2B), leading to early cytotoxicity (Fig. 1B). As 6MP remains available in the medium after exposure to 10 μM 6MP (Table 1), the persistence of abundant 6MP-nucleotide levels (Table 1 and Fig. 4), associated with a complete inhibition of PDNS and a severe depletion of deoxyribonucleotides for DNA synthesis, will prevent further progression through the S phase after 24 hr. This is demonstrated in Fig. 2B at 48 hr: a significant increase of cells in S phase and a decrease of cells in G_2 + M phase. This indicates an arrest of cell progression and cell death in S phase, preventing cell accumulation and cell death in the subsequent G_2 phase.

These data suggest that 6MP should be classified as a class II chemotherapeutic agent [39, 43]: further increase of the 6MP concentration will not increase cytotoxicity after a certain maximum, due to a shift from maximal arrest and cell death in G_2 phase to maximal arrest and cell death in S phase.

The important role of TPMT (EC 2.1.1.67) as an anabolic enzyme with respect to the metabolism of 6MP and the important role of the inhibition of PDNS in the mechanism of 6MP cytotoxicity in our studies is in contrast with the studies of Lennard and co-workers [44, 45]. These authors, studying patients with low dose oral 6MP, could only demonstrate a catabolic effect of TPMT, preventing cytotoxicity of 6MP. The differences between these studies and our data can be explained by the fact that the bioavailability of orally administered 6MP is very low [46, 47] and insufficient to produce adequate Me-tIMP levels in order to inhibit PDNS in residual malignant lymphoblasts in patients during maintenance treatment, whereas our study was performed in malignant lymphoblasts, using high concentrations of 2 and 10 μM 6MP.

Our studies on the cytotoxicity and biochemical

pharmacology of 6MP in human malignant lymphoblasts, using a large number of parameters, provide new insights regarding its mechanism of action, which were unknown until now. These insights, together with the potential synergism of the combination of MTX and 6MP [22, 24] and the excellent penetration of 6MP in the cerebrospinal fluid [32, 33, 46], will be explored in the present study of the Dutch Childhood Leukemia Study Group, using 24 hr intravenous administration of MTX (5 g/m^2) followed by 24 hr intravenous administration of 6MP (1300 mg/m^2) in patients with ALL.

Acknowledgements—The authors wish to thank Dr R. de Graaf for statistical analyses. This work was funded by the Dutch Cancer Society, grant NUKC 82-3.

REFERENCES

1. Elion GB, Burgi E and Hitchings GH, Studies on condensed pyrimidine systems. IX. The synthesis of some 6-substituted purines. *J Am Chem Soc* **74**: 411–414, 1952.
2. Burchenal JH, Murpy ML, Ellison RR, Sykes MP, Tan TC, Leone LA, Karnofsky DA, Craver LF, Dargeon HW and Rhoads CP, Clinical evaluation of a new antimetabolite, 6-mercaptopurine, in the treatment of leukemia and allied diseases. *Blood* **8**: 965–999, 1953.
3. McCollister RJ, Gilbert WR Jr, Ashton DM and Wyngaarden JB, Pseudofeedback inhibition of purine synthesis by 6-mercaptopurine ribonucleotide and other purine analogues. *J Biol Chem* **239**: 1560–1563, 1964.
4. Bennett LL Jr, Brockman RW, Schnebli HP, Chumley S, Dixon GJ, Schabel FM Jr, Dulmadge EA, Skipper HE, Montgomery JA and Thomas HJ, Activity and mechanism of action of 6-methylthiopurine ribonucleoside in cancer cells resistant to 6-mercaptopurine. *Nature* **205**: 1276–1279, 1965.
5. Elion GB, Biochemistry and pharmacology of purine analogues. *Fed Proc* **26**: 898–904, 1967.
6. Tay BS, McC Lilley R, Murray AW and Atkinson MR, Inhibition of phosphoribosyl pyrophosphate amidotransferase from Ehrlich ascites-tumour cells by thiopurine nucleotides. *Biochem Pharmacol* **18**: 936–938, 1969.
7. Montgomery JA, The biochemical basis for the drug actions of purines. *Prog Med Chem* **7**: 69–123, 1970.
8. Bennett LL Jr and Allan PW, Formation and significance of 6-methylthiopurine ribonucleotide as a metabolite of 6-mercaptopurine. *Cancer Res* **31**: 152–158, 1971.
9. Nelson JA and Parks RE Jr, Biochemical mechanisms for the synergism between 6-thioguanine and 6-(methylmercapto)-purine ribonucleoside in sarcoma 180 cells. *Cancer Res* **32**: 2034–2041, 1972.
10. Davidson JD, Studies on the mechanism of action of 6-mercaptopurine insensitive and resistant L1210 leukemia *in vitro*. *Cancer Res* **20**: 225–232, 1960.
11. Warnick CT and Paterson ARP, Effect of methylthioinosine on nucleotide concentrations in L5178Y cells. *Cancer Res* **33**: 1711–1715, 1973.
12. Tidd DM and Paterson ARP, Distinction between inhibition of purine nucleotide synthesis and the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res* **34**: 733–737, 1974.
13. Tidd DM and Paterson ARP, A biochemical mechanism for the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res* **34**: 738–746, 1974.
14. Nelson JA, Carpenter JW, Rose LM and Adamson DJ, Mechanism of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. *Cancer Res* **35**: 2872, 2878, 1975.
15. Woods RA, Henderson RM and Henderson JF, Consequences of inhibition of purine biosynthesis de novo by 6-methylmercaptopurine ribonucleoside in cultured lymphoma L5178Y cells. *Eur J Cancer* **14**: 765–770, 1978.
16. Scannel JP and Hitchings GH, Thioguanine in deoxyribonucleic acid from tumor of 6-mercaptopurine-treated mice. *Proc Soc Biol Med* **122**: 625–629, 1966.
17. Regelson W, Holland JF, Gold GL, Lynch J, Olson KB, Horton J, Hall TC, Krant M, Colsky J, Miller SP and Owens A, 6-Mercaptopurine (NSC-755) given intravenously at weekly intervals to patients with advanced cancer. *Cancer Chem Rep* **51**: 277–282, 1967.
18. Moore GE, Bross IDJ, Ausman R, Nadler S, Jones R Jr, Slack N and Rimm AA, Effects of 6-mercaptopurine (NSC-755) in 290 patients with advanced cancer. Eastern clinical drug evaluation program. *Cancer Chem Rep* **52**: 655–660, 1968.
19. Body GP, Brodovsky HS, Isassi AA, Samuels ML and Freireich EJ, Studies of combination 6-mercaptopurine (NSC-755) and 6-methylmercaptopurine riboside (NSC-40774) in patients with acute leukemia and metastatic cancer. *Cancer Chem Rep* **52**: 315–320, 1968.
20. Adamson PC, Zimm S, Ragab AH, Steinberg SM, Balis F, Kamen BA, Vietti TJ, Gillespie A and Poplack DG, A phase II trial of continuous-infusion 6-mercaptopurine for childhood tumors. *Cancer Chemother Pharmacol* **26**: 343–344, 1990.
21. Zimm S, Johnson GE, Chabner BA and Poplack DG, Cellular pharmacokinetics of mercaptopurine in human neoplastic cells and cell lines. *Cancer Res* **45**: 4156–4161, 1985.
22. Bökkerink JPM, Bakker MAH, Hulscher TW, De Abreu RA and Schretlen EDAM, Purine de novo synthesis as the basis of synergism of methotrexate and 6-mercaptopurine in human malignant lymphoblasts of different lineages. *Biochem Pharmacol* **37**: 2321–2327, 1988.
23. Bökkerink JPM, De Abreu RA, Bakker MAH, Hulscher TW, Van Baal JM, Schretlen EDAM, and De Bruijn CHMM, Effects of methotrexate on purine and pyrimidine metabolism and cell-kinetic parameters in human malignant lymphoblasts of different lineages. *Biochem Pharmacol* **37**: 2329–2338, 1988.
24. Bökkerink JPM, Bakker MAH, Hulscher TW, De Abreu RA, Schretlen EDAM, Van Laarhoven JPRM and De Bruijn CHMM, Sequence-, time-, and dose-dependent synergism of methotrexate and 6-mercaptopurine in malignant human T-lymphoblasts. *Biochem Pharmacol* **35**: 3549–3555, 1986.
25. Bökkerink JPM, De Abreu RA, Bakker MAH, Hulscher TW, Van Baal JM and De Vaan GAM, Dose-related effects of methotrexate on purine and pyrimidine nucleotides and on cell-kinetic parameters in MOLT-4 malignant human lymphoblasts. *Biochem Pharmacol* **35**: 3557–3564, 1986.
26. De Abreu RA, Van Baal JM, Schouten TJ and Schretlen EDAM, High performance liquid chromatographic determination of plasma 6-mercaptopurine in clinically relevant concentrations. *J Chromatogr* **227**: 526–533, 1982.
27. Kapuścinski J and Skoczylas B, Simple and rapid fluorometric method for DNA microassay. *Anal Biochem* **83**: 252–257, 1977.
28. Kufe D, Herrick D, Crumpacker C and Schnipper L, Incorporation of 1-beta-D-arabinofuranosylcytosine into DNA from Herpes Simplex Virus resistant to 9-beta-D-arabinofuranosyladenine. *Cancer Res* **44**: 69–73, 1984.
29. Hollander M and Wolfe DA, *Non Parametric Statistical Methods*, pp. 138–154. Wiley, New York, 1973.

30. Van Baal JM, Van Leeuwen MB, Schouten TJ and De Abreu RA, Sensitive high-performance liquid chromatographic determination of 6-mercaptopurine, 6-thioguanine, 6-mercaptopurine riboside and 6-thioguanine in biological fluids. *J Chromatogr* **336**: 422–428, 1984.
31. Frei III E, Freircich EJ, Gehan E, Pinkel D, Holland JF, Selawry O, Haurani F, Spurr CL, Hayes DM, James GW, Hoogstraten H, Wolman IJ, Traggis DG, Cooper T, Gendel BR, Ebaugh F and Taylor R, From the Acute Leukemia Group B, Studies of sequential and combination antimetabolite therapy in acute leukemia: 6-mercaptopurine and methotrexate. *Blood* **18**: 431–454, 1961.
32. Schouten TJ, De Abreu RA, Schretlen EDAM, Van Baal JM, Van Leeuwen MB and De Vaan GAM, 6-Mercaptopurine: high-dose 24-h infusions in goats. *J Cancer Clin Oncol* **112**: 61–66, 1986.
33. Zimm S, Ettinger LJ, Holcenberg JS, Kamen BA, Vietti TJ, Belasco J, Cogliano-Shutta N, Balis F, Lavi LE, Collins JM and Poplack DG, Phase I and clinical pharmacological study of mercaptopurine administered as a prolonged intravenous infusion. *Cancer Res* **45**: 1869–1873, 1985.
34. Wheeler GP, Bowdon BJ, Adamson DJ and Vail MH, Comparison of the effects of several inhibitors of the synthesis of nucleic acids upon the viability and progression through the cell cycle of cultured H. Ep. no. 2 cells. *Cancer Res* **32**: 2661–2669, 1972.
35. Tomasovic SP, Higashikubo R, Cohen AM and Broom AD, Comparative kinetics of poly(6-thioinosinic acid) and 6-mercaptopurine treated L1210 cells. *Cytometry* **3**: 48–54, 1982.
36. Christie NT, Drake S, Meyn RE and Nelson JA, 6-Thioguanine-induced DNA damage as a determinant of cytotoxicity in cultured chinese hamster ovary cells. *Cancer Res* **44**: 3665–3671, 1984.
37. Lee SH and Sartorelli AC, The effects of inhibitors of DNA synthesis on the cytotoxicity of 6-thioguanine. *Cancer Biochem Biophys* **5**: 189–194, 1981.
38. Barranco SC and Humphrey RM, The effects of beta-2′-deoxythioguanosine on survival and progression in mammalian cells. *Cancer Res* **31**: 583–586, 1971.
39. Hill BT, Cancer chemotherapy. The relevance of certain concepts of cell cycle kinetics. *Biochim Biophys Acta* **516**: 389–417, 1978.
40. Maybaum J and Mandel HG, Unilateral chromatid damage: a new basis for 6-thioguanine cytotoxicity. *Cancer Res* **43**: 3852–3856, 1983.
41. Wotring LL and Roti Roti JL, Thioguanine-induced S and G_2 blocks and their significance to the mechanism of cytotoxicity. *Cancer Res* **40**: 1458–1462, 1980.
42. Loo TL, Luce JK, Sullivan MP and Frei III E, Clinical pharmacologic observations on 6-mercaptopurine and 6-methylthiopurine ribonucleoside. *Clin Pharmacol Ther* **9**: 180–194, 1967.
43. Bruce WR, Meeker BE, Powers WE and Valeriote FA, Comparison of the dose- and time-survival curves for normal and hematopoietic and lymphoma colony-forming cells exposed to vinblastine, vincristine, arabinosylcytosine, and amethopterin. *J Natl Cancer Inst* **42**: 1015–1023, 1969.
44. Lennard L and Lilleyman JS, Variable mercaptopurine metabolism and treatment outcome in childhood lymphoblastic leukemia. *J Clin Oncol* **7**: 1816–1823, 1989.
45. Lennard L, Lilleyman JS, Van Loon J and Weinshilboum RM, Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. *Lancet* **336**: 225–229, 1990.
46. Schouten TJ, De Abreu RA, De Bruijn CHMM, Van Der Kleijn E, Oosterbaan MJM, Schretlen EDAM and De Vaan GAM, 6-Mercaptopurine: pharmacokinetics in animals and preliminary results in children. *Adv Exp Med Biol* **165B**: 367–370, 1984.
47. Zimm S, Collins JM, Riccardi R, O'Neill D, Narang PK, Chabner B and Poplack DG, Variable bioavailability of oral mercaptopurine. Is maintenance chemotherapy in acute lymphoblastic leukemia being optimally delivered? *N Engl J Med* **308**: 1005–1009, 1983.