Biochemical Pharmacology, Vol. 46, No. 9, pp. 1627–1636, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

DESULFURATION OF 6-MERCAPTOPURINE

THE BASIS FOR THE PARADOXICAL CYTOTOXICITY OF THIOPURINES IN CULTURED HUMAN LEUKEMIC CELLS

PETER C. ADAMSON,* FRANK M. BALIS, MARY E. HAWKINS, ROBERT F. MURPHY and DAVID G. POPLACK

Pharmacology and Experimental Therapeutics Section, Pediatric Branch, National Cancer Institute, Bethesda, MD 20892, U.S.A.

(*Received* 5 *April* 1993; *accepted* 23 *June* 1993)

Abstract—The thiopurines have a wide array of effects on purine metabolism, but the primary mechanism of cytotoxicity for both 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) appears to be incorporation of drug into DNA following conversion to the thioguanylate form. In murine leukemic cell lines exposed to a range of thiopurine concentrations *in vitro*, cell survival curves have displayed a phenomenon termed paradoxical cytotoxicity, defined as a decrease in cytotoxicity with increasing drug concentration. The paradoxical cytotoxicity of thiopurines has usually been attributed to concentration-dependent perturbations in the cell cycle. The present study assessed whether the paradoxical cytotoxicity of 6-MP occurred in cultured human leukemic cells, and investigated the biochemical and cell-cycle alterations occurring in these lines at thiopurine concentrations associated with the reverse of cytotoxicity. Paradoxical cytotoxicity was observed in the two human leukemic cell lines examined, but only when 6-MP concentrations exceeded 100 μM. The extent of incorporation of 6-MP metabolites into DNA as thiol- versus non-thiol-containing metabolites was analyzed by performing parallel experiments with ^{14}C- and ^{35}S-radiolabeled drug. With 5 μM 6-MP, approximately 50% of drug was incorporated into DNA as a thionucleotide; however, with increasing drug concentrations, the degree of thionucleotide incorporation remained unchanged or decreased, and the amount incorporated as the desulfurated metabolite (presumably adenylate or guanylate) increased. With 500 μM 6-MP, less than 10% of the drug was incorporated as the thionucleotide. Perturbations in cell cycle reflected the relative amounts of thiol- and non-thiol-containing nucleotide formed at various concentrations of 6-MP. These results suggest that thiopurines may be vulnerable to a unique mechanism of detoxification, in which a human cell can metabolize a cytotoxic drug to a comparatively potent "self-rescue" agent.

The thiopurines 6-mercaptopurine (6-MP)† and 6-thioguanine (6-TG) have been used successfully in the treatment of pediatric and adult hematologic malignancies. Investigations into the intracellular metabolism of thiopurines have revealed a wide array of effects on purine metabolism (for a review, see Ref. 1). In most cell lines, incorporation of drug as the thionucleotide into DNA appears to be the primary mechanism of cytotoxicity for both 6-MP and 6-TG [2–8]. To produce cytotoxicity, 6-MP must be activated intracellularly by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to the nucleotide 6-thioinosine-5′-monophosphate (TIMP) (Fig. 1). TIMP, which can inhibit purine synthesis and interconversion, is subsequently oxidized to thioxanthine monophosphate (TXMP) by IMP dehydrogenase, and then converted to thioguanylate (TGMP) in a glutamine and energy-dependent reaction by guanylate synthetase. After phosphorylation to the triphosphate and reduction to the deoxy- form, this thioguanine nucleotide can be incorporated into DNA. 6-TG is activated via a similar pathway, but does not require ATP-dependent conversion by guanylate synthetase to form a thioguanine nucleotide.

Several investigators have observed a unique phenomenon when evaluating the cytotoxicity of thiopurines in murine cell lines *in vitro*. With increasing concentrations of 6-MP or 6-TG in the culture medium, cell survival initially decreases in a typical concentration-dependent fashion, but then paradoxically increases as the thiopurine concentration is increased further [6, 9–12]. Most investigators have attributed this paradoxical cytotoxicity of thiopurines to concentration-dependent perturbations in the cell cycle. In one study, L1210 murine leukemic cells were arrested in the G_2 phase upon exposure to low concentrations of 6-TG, but at higher drug concentrations, cells were arrested at the G_1-S boundary [11]. These authors attributed the increased cell survival seen at high drug concentrations to arrest at the G_1-S boundary a blockade that would preclude further thiopurine cytotoxicity by interdicting entry into S-phase.

Another mechanism that could account for paradoxical cytotoxicity with increasing drug dose is

* Corresponding author: Peter C. Adamson, M.D., Pharmacology and Experimental Therapeutics Section, Pediatric Branch, National Cancer Institute, Building 10, Room 13N240, 9000 Rockville Pike, Bethesda, MD 20892. Tel. (301) 496-1756; FAX (301) 402-0575.

† Abbreviations: 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; TIMP, 6-thioinosine-5′-monophosphate; TXMP, thioxanthine monophosphate; TGMP, thioguanylate; DTT, dithiothreitol; and TPMT, thiopurine methyltransferase.

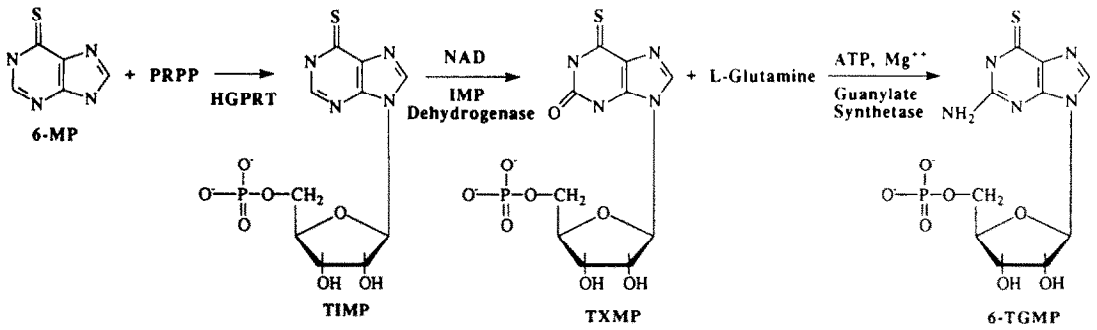

Fig. 1. Pathway for the metabolic activation of 6-mercaptopurine. Thioguanylate formed can subsequently be incorporated into DNA following conversion to the triphosphate form.

concentration-dependent intracellular conversion of a drug into a rescue agent. In the case of 6-MP, loss of the sulfhydryl moiety, from the parent drug to yield hypoxanthine or from an activated thionucleotide form to yield a naturally occurring nucleotide (e.g. TIMP to IMP), could be envisioned as being capable of rescuing cells from 6-MP-induced cytotoxicity.

In the present study, experiments were performed to determine whether the paradoxical cytotoxicity of 6-MP occurred in human leukemic cell lines. Additionally, to determine whether concentration-dependent desulfuration of drug might underlie this phenomenon, we investigated (1) the extent of incorporation of thiol versus non-thiol derivatives of 6-MP into DNA, and (2) the effects of relatively low concentrations of hypoxanthine on 6-MP-induced cytotoxicity and cell-cycle perturbations. Experiments were also performed to determine whether desulfuration of 6-MP occurred prior to or following nucleotide formation.

MATERIALS AND METHODS

Cell lines. MOLT-4 is a human T-cell acute lymphoblastic leukemia line obtained from the American Type Culture Collection (Rockville, MD) and Wilson is a human Burkitt's lymphoma cell line provided by Dr. Ian Magrath of the Pediatric Branch, National Cancer Institute (Bethesda, MD). Both cell lines were maintained and passaged twice weekly in RPMI 1640 + 10% fetal bovine serum at 37° in 5% CO_2 to ensure that cells were in logarithmic phase growth. The doubling times of MOLT-4 and Wilson cells are ~24 and ~19 hr, respectively. For experiments, cells were suspended in RPMI 1640 + 10% charcoal-treated dialyzed fetal bovine serum; the final concentration of hypoxanthine in medium so treated is less than 0.1 μM [13].

Cytotoxicity assay. Except where stated, all chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Thiopurines were dissolved in 0.02 N NaOH and the pH was immediately adjusted to ~8.5 using concentrated HCl.

A previously described modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [14–16] was used to measure cytotoxicity. For each experiment, 135 μL of medium containing 4×10^4 cells/mL were added to wells of a 96-well microtiter plate. Twenty-four hours later, specified concentrations of drug were added to each well, in replicates of 6. Cells were exposed to drug for 48 hr, at which time the number of surviving cells was quantitated with MTT. Cell survival was calculated by subtracting the background absorbance of medium alone at 540 nm (with blanking at 690 nm) and then dividing the absorbance of test wells by the absorbance of the untreated wells. Cytotoxicity experiments using the MTT assay correlate well with the results of clonogenic assays [17, 18], and for the human leukemic cell lines used in the current study yield identical results [19]. Results are the means of at least two separate experiments.

Incorporation of [^{14}C]-6-*MP and* [^{35}S]-6-*MP into DNA.* The amount of 6-MP incorporated into DNA as either the active metabolite thioguanylate, or in a desulfurated form (presumably as adenylate or guanylate), was determined by performing parallel experiments in which cells were incubated with either [^{35}S]-6-MP (Moravek Biochemicals, Brea, CA) or [8-^{14}C]-6-MP (Research Products International, Mount Prospect, IL). For each cell line, 3×10^5 cells/mL were seeded into two sets of three 25-mL flasks and incubated as described above. After ~36 hr of incubation, during which the cells divided logarithmically, 6-MP was added to each set of flasks. For final concentrations of 6-MP of 5, 100 and 500 μM, one set of flasks received [^{14}C]-6-MP at specific activities of 24.9, 8.3 and 2.5 mCi/mmol, respectively, and the other set received [^{35}S]-6-MP at specific activities of 294, 98 and 29.4 mCi/mmol, respectively.

Incorporation of radiolabeled 6-MP into DNA was determined by a modification of a previously described method [20]. Reagent solutions were maintained at 4° and incubations were performed on ice unless otherwise stated. Cells were incubated with drug for 3 hr at 37° in 5% CO_2, washed twice with phosphate-buffered saline (PBS), and then resuspended in 1 mL of 0.3 N NaOH. After 30 min, an equal volume of 20% trichloroacetic acid (TCA) was added and the suspension was incubated for an additional 10 min. Extracts were passed through

Whatman GF/A glass fiber filters and washed with 10% TCA followed by 95% ethanol. Filters were dried, and the bound radiolabel representing macromolecular incorporation was quantitated by liquid scintillation counting (model 2500TR, Packard Instrument Co., Meriden, CT). The single day coefficient of variation (CV) of this method was 10% and the day-to-day CV was 15%. Results shown are the averages of three experiments.

Hypoxanthine modulation of cytotoxicity and cell-cycle perturbations induced by 6-MP. We postulated that desulfuration of 6-MP to a naturally occurring purine could be responsible for the paradoxical cytotoxicity of this thiopurine; it follows that addition of an exogenous source of this naturally occurring purine at maximally cytotoxic concentrations of 6-MP would engender similar effects on the cytotoxicity induced by high concentrations of 6-MP. Hypoxanthine was selected as the exogenous purine as it readily gains intracellular access where it is anabolized to nucleotide forms via the purine salvage pathway. The concentration of hypoxanthine necessary to reverse the cytotoxicity of 6-MP was therefore determined in both of the human cell lines. Twenty-four hours after plating each cell line, hypoxanthine was added at final concentrations ranging from 0.5 to 500 μM. 6-MP at final concentrations of 5, 50 and 500 μM was added simultaneously. Plates were analyzed following 48 hr of drug incubation using the MTT assay.

The effect of 6-MP on cell-cycle distribution was studied in the Wilson cell line. Twenty-four hours after seeding 1×10^5 cells/mL into 25-mL flasks, drug was added to yield final 6-MP concentrations ranging from 5 to 1000 μM. Cells were then incubated with drug for 24 hr prior to analysis, with cells in drug-free medium serving as controls. To determine whether low concentrations of hypoxanthine could modulate the cell-cycle perturbations induced by 6-MP, flasks of cells were incubated simultaneously with 50 μM 6-MP and with 1, 5 or 10 μM hypoxanthine.

For cell-cycle analysis, 24 hr after the addition of drug, cells were washed twice in PBS and prepared for flow cytometry by standard methodology [21]; briefly, 10^7 cells were suspended in 0.3 mL of PBS to which 400 μg of RNAse was added, followed by the addition of 0.5 mL of a 0.01% (w/v) propidium iodide in PBS also containing 0.1% Triton X-100. Cells so prepared were incubated at room temperature for 30 min and then analyzed in the FACScan™ fluorometric system (Becton Dickinson, San Jose, CA) with excitation at 488 nm and emission above 585 nm. Results are the means of two separate experiments.

Desulfuration of 6-MP at the level of the purine base or macromolecule. To determine whether desulfuration of 6-MP occurred prior to or following its anabolism (Fig. 1), experiments were performed using ^{14}C-radiolabeled 6-MP. If desulfuration occurs prior to nucleotide formation, ^{14}C-radiolabeled hypoxanthine would be generated intracellularly promptly after addition of drug; conversely, if desulfuration were to occur following TIMP formation, ^{14}C-radiolabeled hypoxanthine would be expected to appear only at comparatively low concentrations and later times.

To examine this point, 50 mL (2×10^5 cells/mL) from each line were seeded into 75-mL (Costar) flasks and incubated at 37° for 24 hr; at this time [8-^{14}C]-6-MP (sp. act. 1.8 mCi/mmol) was added to yield a final drug concentration of 1000 μM. Intracellular hypoxanthine was quantitated by a modification of a previously described method [22]: briefly, 10-mL aliquots of cells, collected prior to and 2, 3, 4 and 6 hr after addition of drug, were washed twice with ice-cold PBS. Ten microliters of 1 M dithiothreitol (DTT) were added to the cell pellet followed by 500 μL of ice-cold 10% TCA. Following centrifugation, the supernatant was removed and mixed with an equal volume of tri-*n*-octylamine : freon (1 : 4, v/v); then the suspension was centrifuged. The aqueous layer, containing extracted purines, was removed, filtered through a 0.45 μm Millipore ultrafree membrane and injected onto an HPLC consisting of a Waters model 510 pump (Millipore Co., Milford, MA), a Waters WISP 712 automated sample injector, and a 5 μm C_{18} steel column (Beckman Instruments, Inc., San Ramon, CA) equilibrated and developed with a 0.2% acetate buffer containing 1% acetonitrile (v/v) at a flow rate of 1.4 mL/min. Eluant was monitored with a Waters 490 multi-wavelength detector at 254 and 331 nm in line with a radioactive flow detector (Flo-One β, Radiomatic Inc., Tampa, FL). Under these conditions, retention times were approximately 3.8, 4.8 and 5.8 min for 6-thiouric acid, hypoxanthine and 6-MP, respectively. Results are the means of two separate experiments.

RESULTS

Paradoxical cytotoxicity of 6-MP. The cell survival curves for the two human lymphoid cell lines that formed the focus of the present study after exposure to a range of concentrations of 6-MP are shown in Fig. 2. Cell survival initially declined by 2 logarithms as the 6-MP concentration increased from 1 to 100 μM, but then increased in a concentration-dependent fashion at 6-MP concentrations above 100 μM. The magnitude of this paradoxical cytotoxicity was more pronounced in the Wilson cell line.

Incorporation of [^{14}C]-6-MP and [^{35}S]-6-MP into DNA. For both cell lines, the amount of radiolabel incorporated into DNA as non-thiol-containing or thiol-containing nucleotides is illustrated in Fig. 3. The amount of drug incorporated as non-thiol-containing nucleotides was calculated by determining the difference between the amount of ^{14}C- and ^{35}S-radiolabel incorporated. With exposure to 5 μM 6-MP, 40 and 57% of the ^{14}C-label was incorporated as thiol-containing nucleotides in the MOLT-4 and Wilson cell lines, respectively. However, with 500 μM 6-MP, a concentration at which a paradoxical reduction in cytotoxicity was observed, only ~7 and ~8% of the total ^{14}C-label was incorporated as a thiol-containing nucleotide in the MOLT-4 and Wilson cell lines, respectively. The Wilson cell line incorporation of adenylate/guanylate was quantitatively greater at all 6-MP concentrations.

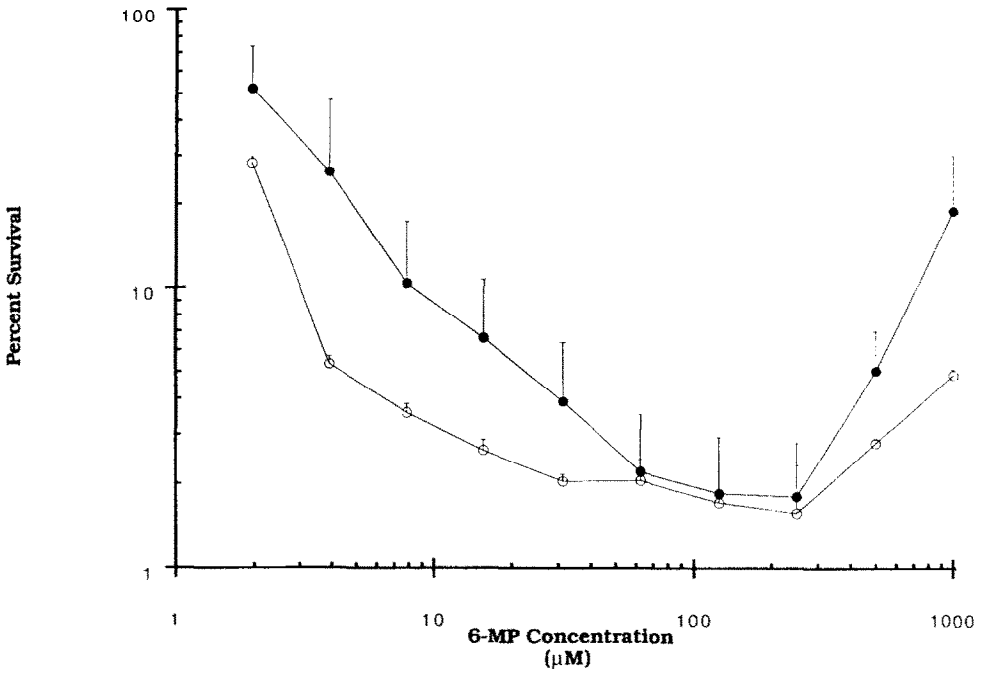

Fig. 2. Paradoxical cytotoxicity of 6-MP in human leukemic cell lines. Survival–concentration curves for MOLT-4 (○) and Wilson (●) cells exposed to increasing concentrations of 6-MP for 48 hr are shown. Values are the means of two experiments, six replicates each; error bars = SD.

The total amount of thiol-containing nucleotides incorporated into DNA did not increase in proportion to the concentration of 6-MP in the medium with either cell line. Thus, in MOLT-4 cells, as the 6-MP concentration increased from 5 to 100 μM, incorporation of thiol-containing nucleotides increased only from 2.1 to 11.7 pmol/10^6 cells. As the 6-MP concentration was further increased from 100 to 500 μM, the amount of thiol-containing nucleotide incorporated into DNA then actually decreased from 11.7 to 3.2 pmol/10^6 cells.

Hypoxanthine modulation of the cytotoxicity and cell-cycle perturbation induced by 6-MP. Cell survival curves for the Wilson cell line co-incubated with 5, 50 or 500 μM 6-MP and a range of hypoxanthine concentrations are illustrated in Fig. 4. Exogenous hypoxanthine engendered a concentration-dependent diminution in 6-MP cytotoxicity at relatively low concentrations. Typically the cytotoxicity from a 6-MP concentration of 500 μM could be partially reversed at exogenous hypoxanthine concentrations as low as 10 μM.

The effects of increasing concentrations of 6-MP on cell-cycle distribution are illustrated in Fig. 5. The most significant perturbation of cell cycle distribution was noted at 50 μM 6-MP. Concentrations higher than this resulted in a progressive normalization in cell cycle distribution such that 1000 μM 6-MP yielded an essentially normal profile. It is noteworthy that low concentrations of exogenously added hypoxanthine (1–10 μM) readily reversed the cell-cycle perturbation induced by 50 μM 6-MP, causing a shift analogous to that produced by increasing concentrations of 6-MP alone (Fig. 6).

Desulfuration of 6-MP: purine base versus nucleotide. Following exposure to [8-^{14}C]-6-MP, 6-thiouric acid was the predominant 6-MP metabolite observed intracellularly (the HPLC method used does not quantitate nucleotides). No ^{14}C-radiolabeled hypoxanthine could be detected. The possibility of low intracellular concentrations of radiolabeled hypoxanthine at the later time points could not be excluded because of interference generated by the large thiouric acid peak.

DISCUSSION

The biochemical pharmacology of the thiopurines is still being intensively researched more than 30 years after they were first synthesized by Elion and co-workers (reviewed in Ref. 1); this time frame illustrates the actual complexity of the intracellular metabolism and diverse actions of these apparently simple purine analogs. Recently, several investigators working with the thiopurines have reported paradoxical cytotoxicity, defined as a decrease in cytotoxicity with increasing drug concentration [6, 9–12]. The two hypotheses proposed to explain this phenomenon are: (1) a thiopurine-induced concentration-dependent blockade in progression through the cell cycle which results in decreased thiopurine incorporation into DNA at high drug concentrations, and (2) 6-MP-induced depletion of intracellular ATP that limits the ATP-dependent conversion of TXMP to TGMP.

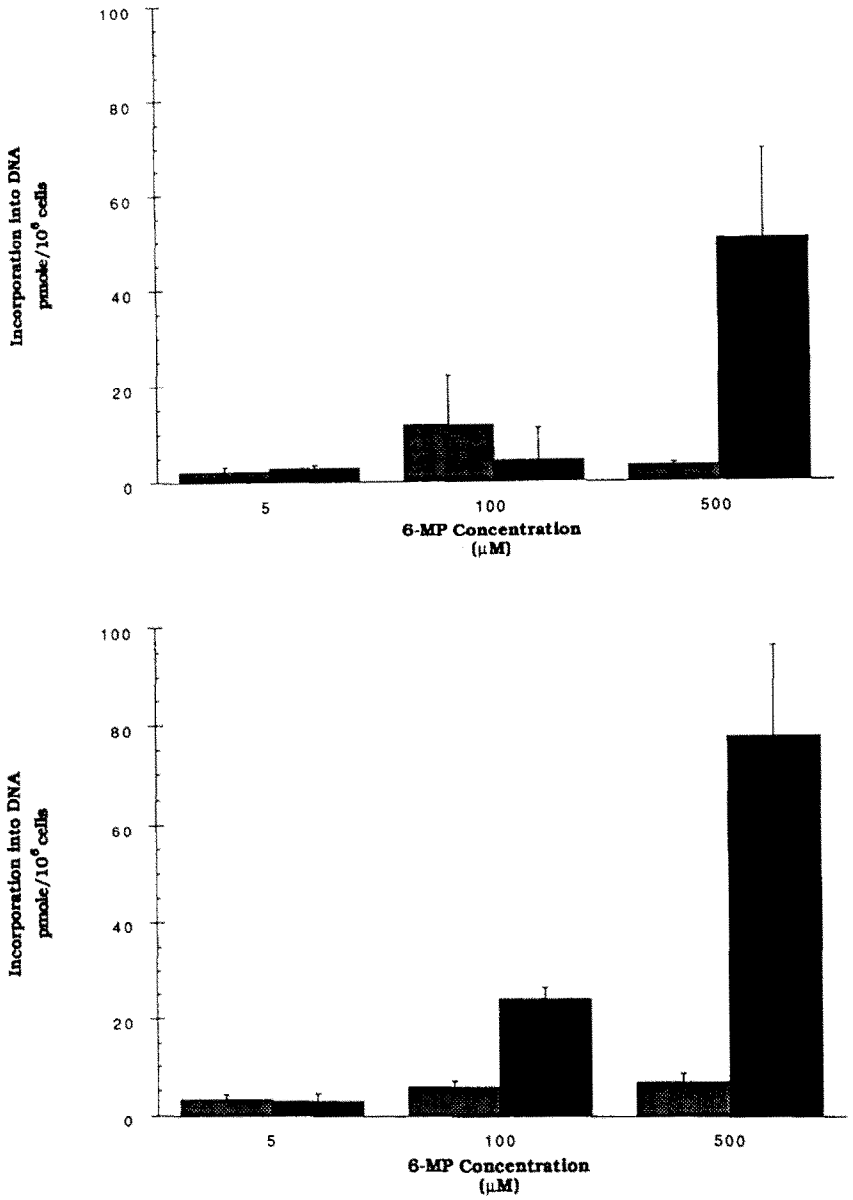

Fig. 3. Incorporation of non-thiol- versus thiol-containing nucleotides into DNA. The amount of drug incorporated into DNA when MOLT-4 cells (top panel) or Wilson cells (bottom panel) were incubated with ^{14}C-labeled 6-MP or ^{35}S-labeled 6-MP is shown. The solid bars (■) indicate the amount of 6-MP incorporated as non-thiol-containing nucleotide (calculated by subtracting the amount of ^{35}S-radiolabel incorporated from ^{14}C-radiolabel incorporated) and the shaded bars (▨) indicate the amount of drug incorporated as thionucleotide. Values are the means ± SD of three experiments.

The data presented here suggest that the paradoxical cytotoxicity of the thiopurines results from desulfuration of drug at high drug concentrations to yield a naturally occurring purine. Such desulfuration not only detoxifies the drug itself, but also generates an antidote in the form of the naturally occurring purine.

Initially, because the activities of enzymes involved in purine metabolism can differ significantly between species [23], we sought to define the concentrations at which paradoxical cytotoxicity of 6-MP was seen in human cell lines. Paradoxical cytotoxicity was observed in both human leukemic cell lines used in the present series of experiments, but only when concentrations of 6-MP exceeded 100 μM (Fig. 2). It should be stressed that this inflection point is significantly higher than the value of 1–5 μM at which reversal in cytotoxicity begins in murine cell lines [9]. Paradoxical cytotoxicity was also observed in both human leukemic cell lines at 6-TG concentrations greater than 100 μM (data not shown).

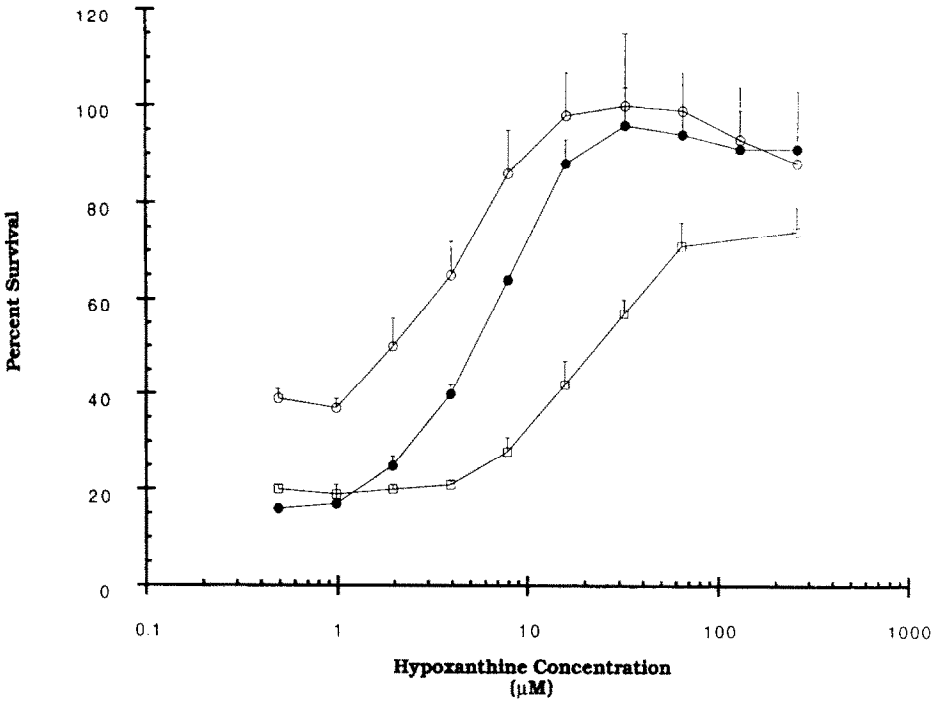

Fig. 4. Reversal of 6-MP-induced cytotoxicity by increasing concentrations of hypoxanthine. Wilson cells were exposed to 5 (○), 50 (●) and 500 (□) μM 6-MP and simultaneously to the indicated concentrations of hypoxanthine. Values are the means of two experiments, six replicates each; error bars = SD.

Several investigators have demonstrated a strong correlation between the amount of thioguanylate incorporated into DNA and the degree of thiopurine cytotoxicity, identifying this mechanism as being more critical than the complex thiopurine-induced perturbations of purine metabolism [3–5]. We have examined the incorporation of 6-MP metabolites into DNA at three concentrations of 6-MP: 5 μM, which falls on the steep declining portion of the cell survival curve; 100 μM, which lies on a plateau where cytotoxicity is maximal; and 500 μM, a concentration at which cell survival increases paradoxically. To determine the amount of 6-MP incorporated into DNA as thioguanylate, human leukemic cell lines were exposed to ^{35}S-labeled 6-MP, and to determine the total amount of 6-MP incorporated as either thioguanylate or as adenylate plus guanylate, parallel experiments were performed with ^{14}C-labeled 6-MP. The difference between the amount of ^{14}C- and ^{35}S-label incorporated was taken to represent the amount of 6-MP first desulfurated and then salvaged prior to DNA incorporation. At the lowest concentration of 6-MP used, a large fraction of drug was incorporated into DNA with its thiol group intact. With increasing drug concentration, however, the degree of thionucleotide incorporation remained essentially unchanged or even decreased, at the same time as the amount incorporated as adenylate or guanylate increases; the end result of the process was that at the highest concentration less than 10% of the drug was incorporated with its thiol group intact.

In the MOLT-4 cell line, the degree of cytotoxicity was correlated closely with the absolute amount of thionucleotide incorporated into DNA rather than with the ratio of thionucleotide to non-thionucleotide incorporated. In the Wilson cell line, however, as the 6-MP concentration was increased from 100 to 500 μM, cell survival increased in parallel despite the fact that incorporation of thionucleotide remained unchanged. One possible explanation for this disparity is that the methodology used in this study could not detect small differences in thionucleotide incorporation (5.8 ± 2.6 pmol/10^6 cells of thionucleotide was incorporated following exposure to 100 μM 6-MP vs 6.8 ± 3.6 pmol/10^6 cells following exposure to 500 μM 6-MP). The lack of correlation could also be a reflection of the shorter incubation time used in the incorporation studies (3 hr) compared with the cytotoxicity studies (48 hr). Alternatively, it may indicate that 6-MP produces cytotoxicity by mechanisms that are different from those responsible for its incorporation into DNA. The paradoxical effect was more prominent in the Wilson cell line, which appears to be more efficient than MOLT-4 cells at desulfurating 6-MP, as demonstrated by the greater proportion of ^{14}C-label relative to ^{35}S-label incorporated at both the 100 and 500 μM concentrations.

It was postulated that events similar to those observed with concentrations of 6-MP that result in paradoxical cytotoxicity could be simulated by addition of an exogenous purine to cells incubated with 6-MP at its maximally cytotoxic concentration.

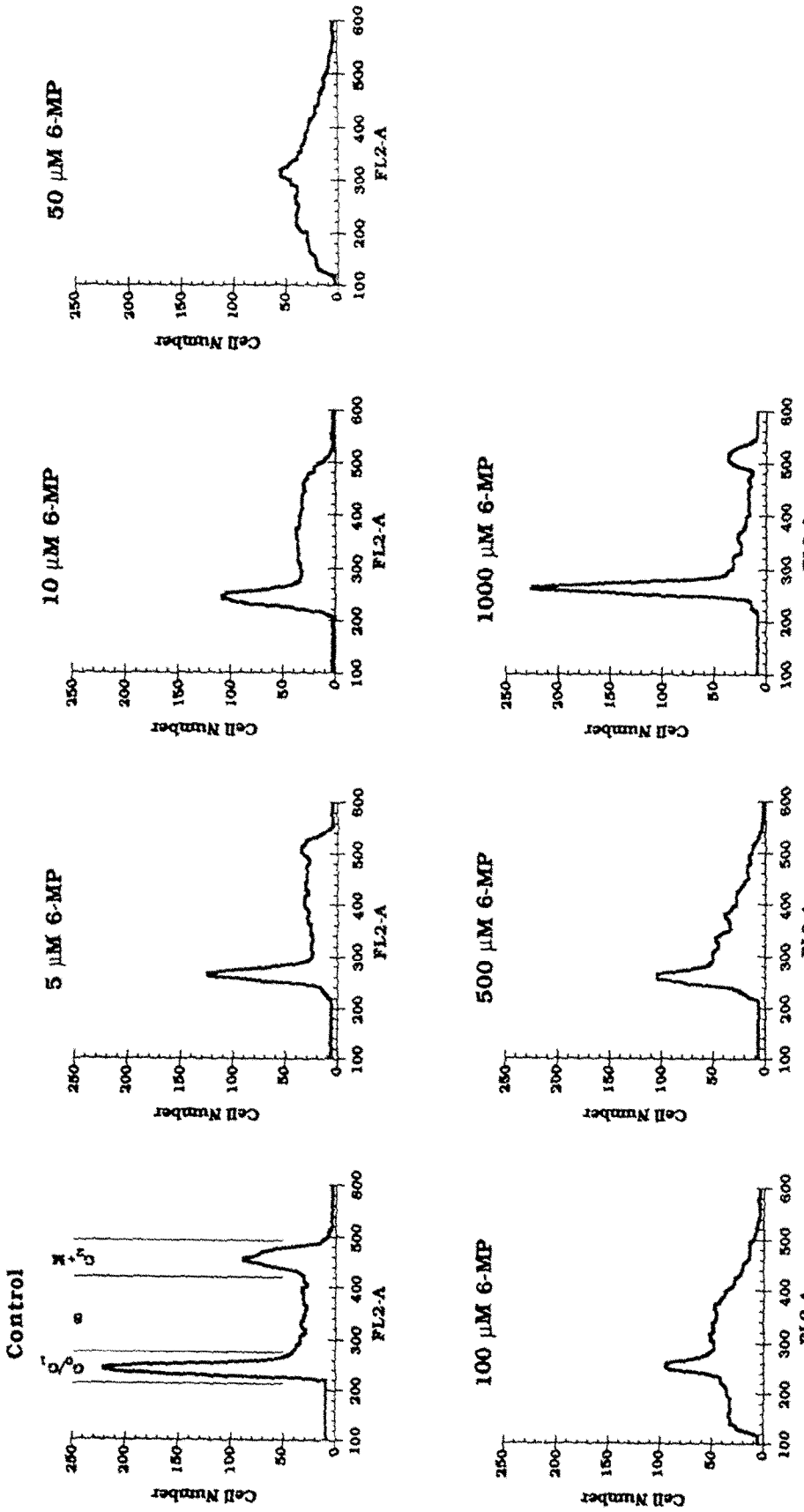

Fig. 5. Effect of increasing concentrations of 6-MP on cell-cycle distribution. The number of cells is shown on the ordinate and the area of the fluorescent signal (measuring red-orange emitted light [FL2-A]) is shown on the abscissa.

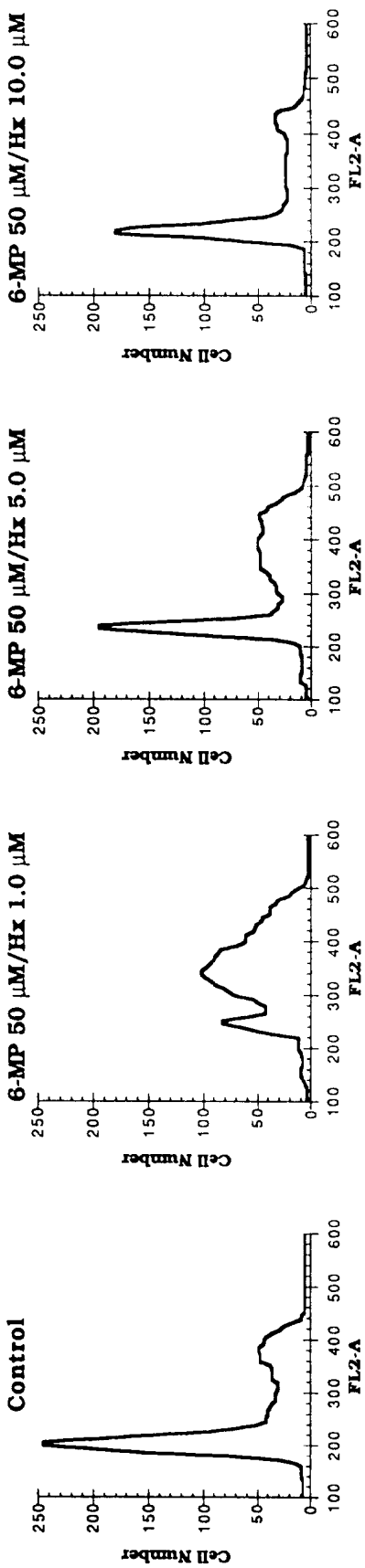

Fig. 6. Effect of low concentrations of hypoxanthine on 6-MP-induced cell-cycle perturbations. The concentration of 6-MP used was 50 μM. The number of cells is shown on the ordinate and the area of the fluorescent signal (measuring red-orange emitted light [FL2-A]) is shown on the abscissa.

Inasmuch as there is currently no inhibitory drug available that will selectively increase intracellular IMP concentrations without perturbing 6-MP metabolism, low concentrations of hypoxanthine were used as an indirect means of increasing intracellular IMP concentrations. Hypoxanthine was shown to antagonize the cytotoxicity of 6-MP in a concentration-dependent fashion. Exogenous hypoxanthine, at concentrations as low as 10 μM, partially reversed the effects of 500 μM 6-MP (Fig. 4), a result that is consistent with previous studies on the influence of the effect of hypoxanthine on thiopurine toxicity in the human HL-60 cell line [13, 24].

Experiments were then performed to determine if desulfuration occurs prior to or following TIMP formation. During the first 6 hr of exposure to [8-^{14}C]-6-MP, no radiolabeled hypoxanthine was detected either intracellularly or in the culture medium (data not shown). This finding is consistent with, but does not prove the conclusion that desulfuration occurs at a step following TIMP formation.

The finding that TIMP formation is required for desulfuration is supported by previous work. In a study with 6-MP resistant *Escherichia coli*, experiments with enzyme extracts revealed that 5-phosphoribosyl-1-pyrophosphate was required for desulfuration of 6-MP, a result supporting the contention that TIMP formation must anticede the dissociation of sulfur. The ability to desulfurate 6-MP was enhanced significantly in drug-resistant bacteria [25]. Similarly, in a study of enzyme extracts from normal and leukemic lymphocytes, TIMP was readily desulfurated under conditions where 6-MP alone was not [26]. The current study made no attempt to determine the phosphorylated form of 6-MP (TIMP, TGMP or their methylated derivatives) susceptible to desulfuration. Indirect evidence is available, however, suggesting that conversion of drug to a 6-methylthiopurine derivative may be a prerequisite to the process. The activity of thiopurine methyltransferase (TPMT), the enzyme responsible for S-methylation of 6-MP, is distributed in populations as a genetic polymorphism, such that 1 in every 300 people exhibit exceedingly low TPMT activity. Pediatric patients with acute lymphoblastic leukemia who have very low TPMT activity are extremely sensitive to the toxic effects of 6-MP, and accumulate increased amounts of thioguanylate within their erythrocytes following administration of standard oral doses of 6-MP [27]; this result suggests that methylation of thiopurines is an important detoxification step. Furthermore, the larger amount of inorganic sulfate generated in patients treated with methylthiopurine compared with patients receiving 6-MP [1] suggests that methylthiopurine formation may be necessary for desulfuration.

It is now possible to address the two previously proposed theories for the paradoxical cytotoxicity of the thiopurines. Disturbances in cell cycle [10–12, 28] have been the most commonly suggested mechanism for this paradox. However, such an attribution is not consistent with the previous work of Skipper *et al.* [29], who demonstrated that, in the case of most S-phase specific drugs, cell survival eventually reaches a plateau, but does not increase, as drug concentration is increased.

The data presented here also do not support the contention that the observed perturbations in cell cycle are the cause of the so-called paradox; rather these perturbations may simply reflect the relative amounts of thiol and non-thiol nucleotide formed at various concentrations of 6-MP. By directly analyzing the cell cycle distribution of MOLT-4 cells exposed to 50 μM 6-MP (maximum cytotoxicity) and various concentrations of hypoxanthine (a direct precursor of IMP), we have observed that as the hypoxanthine concentration increased from 1 to 10 μM, a progressive shift towards a normal cell-cycle distribution occurred. The same phenomenon is observed with 6-MP concentrations that produce paradoxical cytotoxicity, except that the purine nucleotide is then endogenously generated from 6-MP in a concentration-dependent manner.

The second theory to explain the paradoxical reversal of the cytotoxicity of 6-MP is that ATP depletion, as a direct consequence of exposure to the drug, interrupts the ATP-dependent reaction with guanylate synthetase [9]; this conjecture has several significant drawbacks. First, a great many cellular processes critical to cell survival are ATP dependent, so that depletion of intracellular ATP to a limiting level would very likely result in cell death, not survival. Second, the magnitude of the decrease in intracellular ATP concentrations is independent of the 6-MP concentration used; in other words, the decrease in intracellular ATP observed with 6-MP concentrations on the steep portion of the cell survival curve is similar to that observed with 6-MP concentrations that produce paradoxical increase in survival. Finally, an alternative theory would have to be proposed to explain the paradoxical cytotoxicity of 6-TG, inasmuch as ATP is not required for its conversion into thioguanylate.

The *in vitro* observations of paradoxical thiopurine cytotoxicity made prior to this study have been based on studies with murine cell lines with purine pathway capabilities known to differ quantitatively from those of human cells. Put otherwise, the drug concentrations required for paradoxical cytotoxicity in cultivated human cell lines are significantly higher than those required by murine cell lines, and exceed 6-MP concentrations of 100 μM. Clinically, this concentration is never achieved following either oral or continuous intravenous infusion of 6-MP [30, 31]. Thus, we find no evidence to support the concept of dose reduction for patients treated with 6-MP to avoid a paradoxical reversal of cytotoxicity and efficacy. Our previous observations using human leukemic cell lines suggest that continuous exposure to 1–10 μM concentrations of 6-MP remains an appropriate pharmacokinetic target [14].

Several principles emerge from the studies presented here. The enzymatic capabilities of the metabolic steps responsible for the anabolism of a given drug must be considered when interpreting the effects of exposing cells to increasing concentrations of the drug. The paradoxical reversal of the cytotoxicity of 6-MP probably occurs after saturation of one or more anabolic steps, leading in turn to a disproportionate increase in desulfuration of

the thiopurine with increasing 6-MP concentrations. This desulfuration pathway requires additional study, as it is a potential site of drug resistance to thiopurines [26]. Moreover, it is now clear that up-regulation of the pathway could theoretically diminish or eliminate the cytotoxic potential of this entire class of agents. Our results suggest that thiopurines may be vulnerable to a unique mechanism of detoxification, in which a malignant cell can eliminate a cytotoxic drug and simultaneously produce a potent autogenous antidote.

Acknowledgement—We thank Drs. David Johns and David Cooney for their insightful comments and critical review of this work.

REFERENCES

1. Elion GB, Symposium on immunosuppressive drugs. Biochemistry and pharmacology of purine analogues. *Fed Proc* **26**: 898–904, 1967.
2. Horakova K, Kalafut F, Navarova J and Paterson ARP, Delayed expression of 6-mercaptopurine cytotoxicity toward HeLa cells. *Neoplasma* **21**: 555–560, 1974.
3. Tidd DM and Paterson ARP, A biochemical mechanism for the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res* **34**: 738–746, 1974.
4. Tidd DM and Paterson ARP, Distinction between inhibition of purine nucleotide synthesis and the delayed reaction of 6-mercaptopurine. *Cancer Res* **34**: 733–737, 1974.
5. Nelson JA, Carpenter JW, Rose LM and Adamson DJ, Mechanisms of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. *Cancer Res* **35**: 2872–2878, 1975.
6. 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.
7. Lee SH and Sartorelli AC, The effects of inhibitors of DNA biosynthesis on the cytotoxicity of 6-thioguanine. *Cancer Biochem Biophys* **5**: 189–194, 1981.
8. Maybaum J and Mandel HG, Unilateral chromatid damage: A new basis for 6-thioguanine cytotoxicity. *Cancer Res* **43**: 3852–3856, 1983.
9. Liliemark J, Pettersson B, Engberg B, Lafolie P, Masquelier M and Peterson C, On the paradoxically concentration-dependent metabolism of 6-mercaptopurine in WEHI-3b murine leukemia cells. *Cancer Res* **50**: 108–112, 1990.
10. Matsumura S, Hoshino T, Weizsaecker M and Deen DF, Paradoxical behavior of 6-mercaptopurine as a cytotoxic agent: Decreasing cell kill with increasing drug dose. *Cancer Treat Rep* **67**: 475–480, 1983.
11. Maybaum J, Morgans CW and Hink LA, Comparison of *in vivo* and *in vitro* effects of continuous exposure of L1210 cells to 6-thioguanine. *Cancer Res* **47**: 3083–3087, 1987.
12. Moses K, Willmore E, Harris AL and Durkacz BW, Correlation of enhanced 6-mercaptopurine cytotoxicity with increased phosphoribosylpyrophosphate levels in Chinese hamster ovary cells treated with 3-aminobenzamide. *Cancer Res* **50**: 1992–1996, 1990.
13. Zimm S, Johnson GE and Poplack DG, Modulation of thiopurine cytotoxicity in the HL-60 cell line by physiological concentrations of hypoxanthine. *Cancer Res* **46**: 6286–6289, 1986.
14. Adamson PC, Balis FM, Arndt Ca, Holcenberg JS, Narang PK, Murphy RF, Gillespie AJ and Poplack DG, Intrathecal 6-mercaptopurine: Preclinical pharmacology, phase I/II trial, and pharmacokinetic study. *Cancer Res* **51**: 6079–6083, 1991.
15. Mossman T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1983.
16. Twentyman PR and Luscombe M, A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* **56**: 279–285, 1987.
17. Plumb JA, Milroy R and Kaye SB, Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res* **49**: 4435–4440, 1989.
18. Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB, Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res* **47**: 936–942, 1987.
19. Adamson PC, Balis FM, Cole D and Poplack DG, Comparison of nonclonogenic assays to clonogenic assays for chemosensitivity testing of leukemic lymphoblasts. *Proc Am Assoc Cancer Res* **32**: 385, 1991.
20. Zerler B, Roberts RJ, Mathews MB and Moran E, Differential functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. *Mol Cell Biol* **7**: 821–829, 1987.
21. Tate EH, Wilder ME, Cram LS and Wharton W, A method for staining 3T3 cell nuclei with propidium iodide in hypotonic solution. *Cytometry* **4**: 211–215, 1983.
22. Khym JX, Analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. *Clin Chem* **21**: 1245–1252, 1975.
23. Scholar EM and Calabresi P, Identification of the enzymatic pathways of nucleotide metabolism in human lymphocytes and leukemia cells. *Cancer Res* **33**: 94–103, 1973.
24. Hashimoto H, Kubota M, Shimizu T, Takimoto T, Kitoh T, Akiyama Y and Mikawa H, Biochemical basis of the prevention of 6-thiopurine toxicity by the nucleobases, hypoxanthine and adenine. *Leuk Res* **14**: 1061–1066, 1990.
25. Coggin J, Loosemore M and Martin W, Metabolism of 6-mercaptopurine by resistant *Escherichia coli* cells. *J Bacteriol* **92**: 446–454, 1966.
26. Martin W, Crichton I, Yang R and Evans A, The metabolism of thioinosinic acid by 6-mercaptopurine sensitive and resistant lymphocytes. *Proc Soc Exp Biol Med* **140**: 423–428, 1972.
27. Lennard L, Lilleyman JS, Loon JV and Weinshilboum RM, Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukemia. *Lancet* **336**: 225–229, 1990.
28. Maybaum J, Ting P and Rogers CE, Prediction of thioguanine-induced cytotoxicity by dual-parameter flow cytometric analysis. *Cancer Chemother Pharmacol* **24**: 291–294, 1989.
29. Skipper HE, Schabel FM, Mellett LB, Montgomery JA, Wikoff LJ, Lloyd HH and Brockman RW, Implications of biochemical, cytokinetic, pharmacologic, and toxicologic relationships in the design of optimal therapeutic schedules. *Cancer Chemother Rep* **54**: 431–450, 1970.
30. 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.
31. Zimm S, Ettinger LJ, Holcenberg JS, Kamen BA, Vietti TJ, Belasco J, Cogliano N, Balis FM, Lavi LE, Collins JM and Poplack DG, Phase I and clinical pharmacological study of 6-mercaptopurine administered as a prolonged intravenous infusion. *Cancer Res* **45**: 1869–1873, 1985.