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6-Ethylmercaptopurine-mediated growth inhibition of HL-60 cells in vitro irrespective of purine salvage*

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Summary. A variety of purine analogs inhibit the growth and induce the differentiation of human promyelocytic leukemia (HL-60) cells that lack the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Mechanisms by which purine analogs induce differentiation offer unique potential for cancer chemotherapy. The guanine analogs, 6-thioguanine and 8-azaguanine, induce granulocytic differentiation of HGPRT-deficient HL-60 promyelocytes. Although these compounds are useful as model purine analogs that induce differentiation in HGPRT-deficient HL-60 cells, they suffer the disadvantage that they are highly cytotoxic to wild-type cells. We studied the effect of the hypoxanthine analog 6-ethylmercaptopurine on wild-type and HGPRT-deficient HL-60 cells. 6-Ethylmercaptopurine inhibits growth and produces a specific terminal end-cell in both types of HL-60 cells. The mechanism appears to be independent of the normal modes of cytotoxic activation through HGPRT or adenine phosphoribosyltransferase (APRT), since no new peaks were seen in HPLC chromatograms of the nucleotide pools. Furthermore, hypoxanthine and adenine failed to prevent growth inhibition by 6-ethylmercaptopurine, and inhibition of IMP dehydrogenase and the consequential alteration of the guanine nucleotide pools does not appear to be involved. The mechanism differs from that of guanine analog-induced differentiation in HGPRT-deficient HL-60 cells.

Introduction

The idea of using purine antimetabolites as the basis for chemotherapeutic intervention for the treatment of cancer was first put forth in the 1950s [4]. The pharmacologic mode of action for these antimetabolites is the anabolic conversion of these compounds to their corresponding nucleotides. Incorporation into nucleic acids results in the inhibition of DNA and RNA synthesis and thus kills proliferating cells. Conversion of 6-thioguanine (TG) and 6-mercaptopurine (MP) into 6-thioguanosine 5'-monophosphate by salvage enzymes results in significant cytotoxicity to proliferating cells [3, 19].

Cells that are resistant to TG and MP due to a deficiency in the salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) still exhibit growth inhibition by these compounds. Murine erythroleukemia (MEL) and human promyelocytic leukemia (HL-60) cells have been shown to undergo both functional and morphological differentiation on treatment with TG and MP [2, 7]. Significant cytotoxicity with very little differentiation is observed on treatment of wild-type HL-60 cells with TG and MP [18]. However, HGPRT-deficient HL-60 cells could be induced to differentiate following treatment with a variety of guanine or hypoxanthine analogs [2, 16, 18]. That cytotoxic metabolites are not responsible for this induction of differentiation was first suggested by Gusella and Housman [7]. Further studies concluded that induction of differentiation is directly due to the nucleobase analog and not some metabolite [8].

Insight into the mechanism of how the nucleobase form of TG is responsible for differentiation was recently shown by Kretz et al. [11]. 6-Thioguanine was found to be incorporated into the anticodon of tRNA in place of the normal substrate queuine, also a highly modified guanine analog, in HGPRT-deficient HL-60 cells. The resulting structural change in tRNA, which may alter codon-anticodon interactions during protein synthesis, is the likely molecular basis for induction of differentiation, since queuine (at an 800-fold lower concentration than TG) could reverse both the tRNA structural change and the induction of differentiation. The hypoxanthine analog MP also induces HGPRT-deficient HL-60 cells to differentiate. However, MP is neither an inhibitor [5] nor a substrate [19] for the queuine modification enzyme, thus indicating that multiple epigenetic mechanisms may exist by which nonactivated nucleobase analogs will induce cellular differentiation.

The possibility of using epigenetic targets for chemotherapeutic drug intervention is an attractive one, since the toxic side effects associated with the

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Abbreviations: e^6MP, 6-ethylmercaptopurine; TG, 6-thioguanine; MP, 6-mercaptopurine; AG, 8-azaguanine; MEL, murine erythroleukemia; FBS, fetal bovine serum; queuine, 7-5-[(1S,4S,5R)-4,5-dihydroxy-2-cyclopenten-1-yl)amino]methyl-7-deazaguanine; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; TCA, trichloroacetic acid

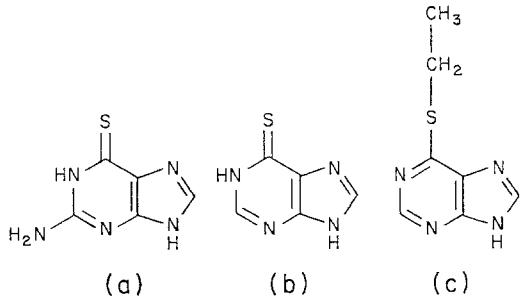

Fig. 1. Structures of **a** TG, **b** MP, and **c** e^6MP

chemotherapy may be reduced or eliminated. This mode of action could prove to be especially beneficial in cancers that do not have high proliferation rates and thus do not require metabolic activation of the compound for its efficacy. In this study, we compare the hypoxanthine analog 6-ethylmercaptopurine (e^6MP) with TG and MP for its ability to inhibit the growth of both wild-type and HGPRT-deficient HL-60 cells. The structures of the analogs evaluated are shown in Fig. 1.

Materials and methods

Cell culture. Wild-type HL-60 cells obtained from Dr. Robert Gallo at the National Cancer Institute and HGPRT-deficient HL-60 cells provided by Dr. Linda Thompson, Scripps Clinic and Research Foundation, were grown in suspension culture at 37° C in a humidified atmosphere with 10% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

Growth inhibition studies. The effects of the analogs on the growth of HGPRT-deficient and wild-type HL-60 cells were monitored. Cells were taken from early stationary-phase cultures and diluted to a concentration of 1×10^5 cells/ml in 25-cm^2 flasks. The cells were counted daily using a Coulter Counter model ZM.

[^{3}H]-Hypoxanthine and [^{3}H]-adenine incorporation assay. For evaluation of analog effects, proliferating wild-type HL-60 cells (1×10^7) were harvested by centrifugation and resuspended in 5 ml RPMI-1640 medium. A 1-ml sample of the cell suspension was added to each of five tubes containing 1 ml of the following in RPMI-1640 medium: no addition, 0.8 m*M* TG, 0.8 m*M* MP, or 0.8 m*M* e^6MP. Therefore, the final concentration of each analog was 0.4 m*M*. Next, 1 μCi [^{3}H]-hypoxanthine or [^{3}H]-adenine was added to each of the tubes, after which the cells were incubated at 37° C for 1 h. Incorporation of radiolabelled hypoxanthine or adenine was determined by trichloroacetic acid (TCA) precipitation.

Nucleotide analysis. Nucleotides were extracted from cells into 2 *M* perchloric acid and prepared for HPLC analysis as previously described [6]. Neutralized cell extract was placed on a Whatman PX5 10/25 SAX column and eluted at a flow rate of 2 ml/min with buffer A (0.005 *M* KH_2PO_4, pH 4.5) and a 1%/min gradient of buffer B (0.75 *M* KH_2PO_4, pH 4.5) for 20 min, followed by 2% buffer B/min for 17.5 min and, finally, 55% buffer B for 5 min. Quantitation was based on peak area using an Altex CR2-A integrating recorder and external standards. Nucleotide levels were corrected for cell protein content.

[^{3}H]-Dihydroqueuine incorporation assay. This assay was carried out to determine whether MP and e^6MP were inhibiting queuine modification of tRNA, and it represents a modification of a procedure developed for evaluating inhibitors of the queuine modification in cells in monolayer culture [15]. HGPRT-deficient HL-60 cells (1 10^7) were harvested by centrifugation and resuspended in 6 ml RPMI-1640 medium containing 10% dialyzed FBS for 24 h. Because cells in vitro must obtain queuine from the serum that supplements the culture medium [9], the use of dialyzed FBS generates queuine hypomodified tRNA, the required substrate for the assay. Cell suspension (0.5 ml) was then added to each of 11 tubes containing the analogs at various concentrations, along with radiolabelled dihydroqueuine (final concentration, 0.15 μ*M*) in 0.5 ml RPMI-1640 medium. The cells were incubated for 4 h at 37° C. The amount of dihydroqueuine incorporation into the tRNA was then determined by TCA precipitation of the lysed cells.

Materials. FBS was purchased from Sterile Systems (Logan, Utah), and dialyzed FBS was prepared by dialysis at 4° C against 15–20 volumes of Hanks' balanced salt solution, with four changes over a period of 45 h. The dialyzed FBS final volume, 7% greater than the starting volume, was then filter-sterilized. RPMI-1640 medium was purchased from GIBCO (Grand Island, NY), and TG, MP, e^6MP, NBT, Wright-Giemsa stain, and 12-o-tetradecanoyl-phorbol-13-acetate were purchased from Sigma Chemical Company (St. Louis, Mo). [^{3}H]-Hypoxanthine (6.7 Ci/mmol) was obtained from Amersham (Arlington Heights, Ill). [^{3}H]-Dihydroqueuine (3.5 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, Mass), the radiolabelled reduced queuine being custom-generated by catalytic reduction of 6 mg queuine with tritium gas. Queuine (purified from bovine amniotic fluid [10] was supplied by Dr. Jon R. Katze, University of Tennessee Health Sciences Center (Memphis, Tenn).

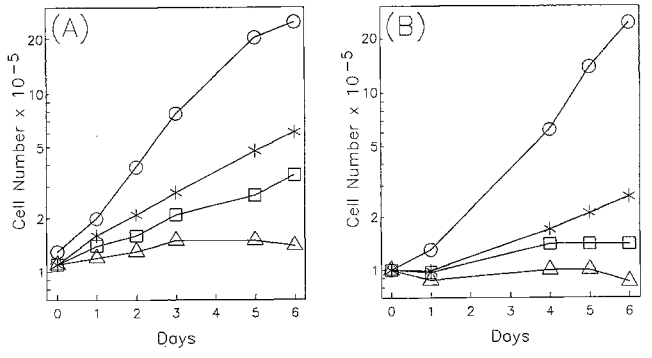

Fig. 2. Dose-dependent growth inhibition for A HGPRT-deficient and **B** wild-type HL-60 cells treated with e^6MP. The cell populations were grown in the absence or presence of e^6MP as follows: none (○), 0.2 mM (×), 0.4 mM (□), and 0.6 mM (△)

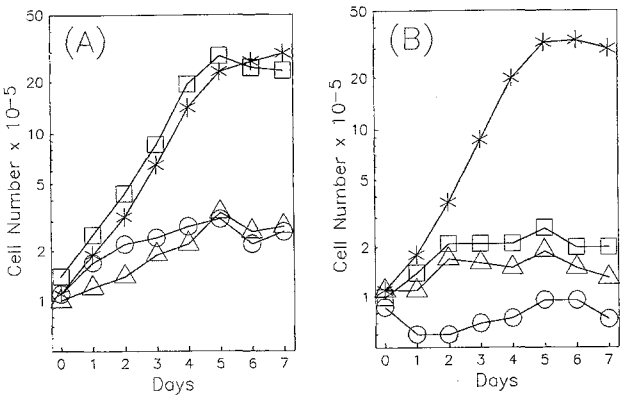

Fig. 3. Growth curves for HGPRT-deficient and wild-type HL-60 cells treated with purine analogs. A HGPRT-deficient HL-60 cells were treated as follows: untreated controls (∗), 0.6 m*M* TG (○), 0.6 m*M* MP (□), and 0.6 m*M* e^6MP (△). **B** Wild-type HL-60 cells were treated as follows: untreated controls (∗), 0.6 m*M* TG (○), 0.6 m*M* MP (□), and 0.6 m*M* e^6MP (△) (s)

Results

A dose-dependent inhibition of growth was observed when HGPRT-deficient or wild-type HL-60 cells were treated with increasing concentrations of e^6MP (Fig. 2). In the experiment illustrated in Fig. 2 A, HGPRT-deficient cells were treated with 0.2, 0.4, and 0.6 m*M* e^6MP, and in that described in Fig. 2 B, wild-type cells were treated with the same concentrations of this analog. A comparable dose dependence was observed for TG with HGPRT-deficient HL-60 cells, whereas the optimal concentration of MP for growth inhibition of HGPRT-deficient HL-60 cells was between 3.0 and 4.0 m*M* (data not presented).

The optimal concentration for e^6MP was then used to compare the growth inhibition of e^6MP, MP, and TG in both HGPRT-deficient and wild-type HL-60 cells (Fig. 3). In HGPRT-deficient cells at a concentration of 0.6 m*M*, MP had no effect on cell growth (Fig. 3 A). However, e^6MP equaled the ability of TG to inhibit cell growth at this concentration. The viability of these cells on day 4 was quite good for both e^6MP (0.6 m*M*, 68%) and TG (0.6 m*M*, 60%). In wild-type cells, MP and TG were highly cytotoxic, with no viability on day 4 (Fig. 3 B). In contrast, wild-type cells treated with e^6MP maintained high viability on day 4 (65%), and the level of growth inhibition was equal to that seen in HGPRT-deficient HL-60 cells.

Inhibition of cell growth by e^6MP and simultaneous maintenance of high viability suggests the possibility that this analog was inducing cell differentiation. TG and MP are known to induce morphological differentiation in HGPRT-deficient HL-60 cells down the granulocytic pathway. Because e^6MP was equally effective in both wild-type and HGPRT-deficient HL-60 cells and also maintained a high degree of viability, the morphological changes in these cells were evaluated (Fig. 4).

HGPRT-deficient HL-60 cells treated for 7 days (Fig. 4 A) were examined with Wright-Giemsa stain. As expected, TG induced differentiation of the HGPRT-deficient cells toward greater maturity down the granulocytic pathway. Differential counting showed that approximately 50% of the cells consisted of myelocytes and metamyelocytes (Fig. 4 B). At a concentration of 0.6 m*M*, MP had no effect on morphology (Fig. 4 C). No evidence of granulocytic maturation was seen with e^6MP (Fig. 4 D). The morphology of this end cell was characterized by a larger cell size, increased cytoplasmic vacuole formation, and large, segmented nuclei. The same type of terminal end-cell was observed for the wild-type cells. These characteristics suggested aberrant maturation of these cells down the monocytic/macrophage pathway. Preliminary evidence indicates that cells treated with e^6MP are positive for nonspecific esterase activity, a characteristic of monocytic maturation (data not shown).

As described above, e^6MP was moderately cytotoxic to both wild-type and HGPRT-deficient HL-60 cells. However, both cell types showed an increased sensitivity to e^6MP in dialyzed serum. To ascertain whether this increased sensitivity was due to the absence of protective purines in dialyzed serum, exogenous hypoxanthine (1 m*M*) and adenine (0.2 m*M*) were added in the presence of e^6MP (0.1 m*M* and 1.0 m*M*). Neither hypoxanthine nor adenine could inhibit the cytotoxicity of e^6MP to wild-type HL-60 cells (data not shown).

To determine if the effect of e^6MP is mediated through the purine salvage pathway, analog inhibition of

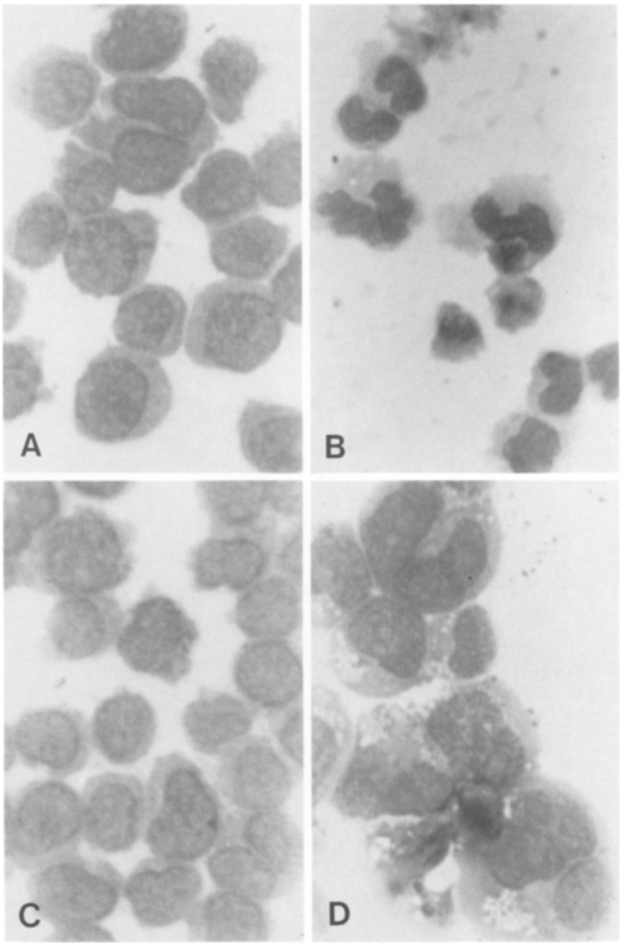

Fig. 4. Photomicrographs of control and analog-treated HGPRT-deficient HL-60 cells stained for differential counts. **A** untreated control cells, **B** TG-treated cells, **C** MP-treated cells, **D** e^6MP-treated cells. The analog treatments were carried out at 0.6 m*M* for 7 days (from Fig. 3 A). The cells were stained with Wright-Giemsa as described in *Materials and methods*

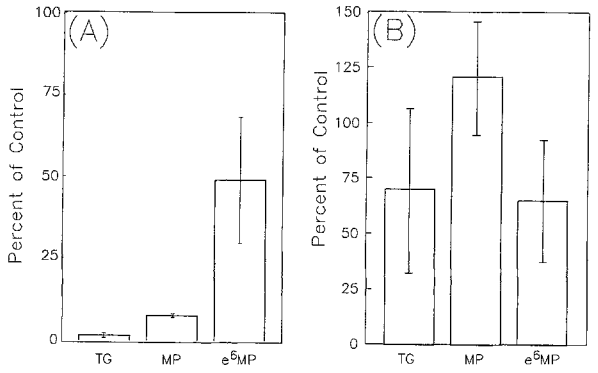

Fig. 5. Analog inhibition of **A** [^{3}H]-hypoxanthine and **B** [^{3}H]-adenine incorporation into nucleic acids by wild-type HL-60 cells. Cells were treated with 0.4 m*M* TG, MP, or e^{6}MP, and the mean of three independent experiments (±SD) is presented as the percentage of untreated control incorporation

radiolabelled hypoxanthine and adenine incorporation into wild-type HL-60 cells was compared (Fig. 5). TG and MP had significant effects on radiolabelled hypoxanthine incorporation, whereas e^{6}MP had a much weaker effect (Fig. 5 A). TG and e^{6}MP did not significantly inhibit radiolabelled adenine incorporation, whereas MP appeared to stimulate incorporation (Fig. 5 B).

To determine if e^{6}MP treatment altered purine nucleotide pools, nucleotides extracted from wild-type cells treated with e^{6}MP were then analyzed by HPLC (Fig. 6). No changes were observed in the guanine or adenine nucleotides between control cells and cells treated with 0.6 m*M* e^{6}MP. However, a decrease (40%) was observed in the uridine triphosphate (UTP) concentration in cells treated with e^{6}MP.

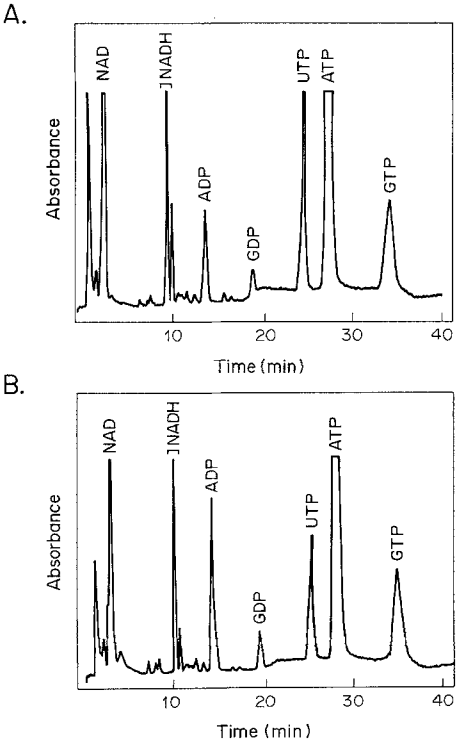

Fig. 6. HPLC profiles of ribonucleotides from **A** control wild-type HL-60 cells and **B** cells treated with 0.6 m*M* e^{6}MP for 2 h

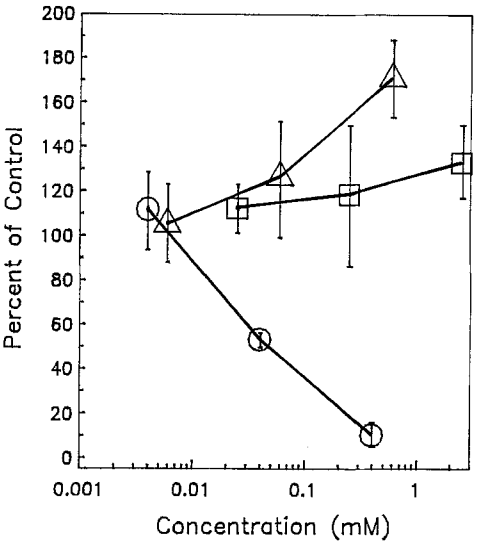

Fig. 7. Analog inhibition of [^{3}H]-dihydroqueuine incorporation into tRNA by HGPRT-deficient HL-60 cells. Cells were treated with various concentrations of TG (○), MP (□), or e^{6}MP (△) as described in *Materials and methods.* The mean of three independent experiments (±SD) is presented as the percentage of untreated control incorporation

TG is a substrate for the tRNA modification enzyme tRNA-guanine ribosyltransferase and is inserted in place of the normal base queuine. An assay evaluating analog inhibition of radiolabelled dihydroqueuine incorporation in HGPRT-deficient HL-60 cells was carried out to determine whether the hypoxanthine analogs MP and e^{6}MP were substrates/inhibitors of queuine modification of tRNA. A significant, dose-dependent inhibition of radiolabel incorporation was observed after treatment with TG, whereas no inhibition was seen with MP or e^{6}MP treatment (Fig. 7). The highest concentration used for each analog was comparable with that required to induce differentiation of these cells.

Discussion

Early strategies for using nucleobase analogs such as TG and MP in chemotherapy have been based on their metabolic activation into cytotoxic compounds inhibiting DNA and RNA synthesis [1, 3]. However, cells that are resistant to these nucleobase analogs due to their lack of the purine salvage enzyme HGPRT can be induced to differentiate when exposed to higher concentrations of the same agents [2, 16, 18]. The mechanism by which these analogs induce cellular differentiation is of interest in the design of novel pharmacologic agents for the treatment of cancer. The disadvantage of analogs such as TG and MP is that their differentiating properties are observed only under special conditions. Although useful for studying the mechanism by which these compounds induce differentiation, they are extremely cytotoxic to normal cells. In this regard, we evaluated the growth inhibition of wild-type and HGPRT-deficient HL-60 cells by e^{6}MP.

The growth inhibition observed with e^{6}MP was dose-dependent in both wild-type and HGPRT-deficient HL-60

cells (Fig. 2). Evaluations of all three analogs (TG, MP, and e^6MP) at the optimal concentration for e^6MP (0.6 m*M*) in both cell types were also of interest (Fig. 3). Although TG and MP were cytotoxic to wild-type cells, as previously reported [18], e^6MP could inhibit cell growth in both wild-type and HGPRT-deficient cells with no cytotoxic effects (Fig. 3).

Morphological analyses (Fig. 4) indicated that e^6MP did not induce the granulocytic maturation in HGPRT-deficient HL-60 cells that is found when these cells are treated with TG. However, the terminal end-cell produced by e^6MP is the same for both wild-type and HGPRT-deficient HL-60 cells. The morphological changes found in these cells after e^6MP treatment are characterized by an increase in cell size, increased cytoplasmic vacuole formation, and an increased size and segmentation of the nucleus. Although promyelocytes are normally committed to granulocytic differentiation, HL-60 cells can be induced down both the granulocytic and monocytic pathways by a variety of reagents [2, 17]. Some of the morphological characteristics observed in cells treated with e^6MP suggested aberrant monocytic differentiation. Preliminary evidence indicates that cells treated with e^6MP are positive for nonspecific esterase activity (data not shown), a characteristic of monocytic maturation [12]. A more detailed analysis of monocyte-specific markers is currently being undertaken.

The possibility that growth inhibition was due to purine salvage of e^6MP was tested by monitoring the inhibition of radiolabelled hypoxanthine and adenine incorporation (Fig. 5). The absence of HGPRT activity in the mutant HL-60 cells argues against this purine salvage enzyme's playing a role in the action of e^6MP, although the analog did somewhat inhibit hypoxanthine salvage in wild-type cells. In addition, slight inhibition of adenine salvage by both TG and e^6MP argues against a role for adenine phosphoribosyltransferase (APRT) in the cellular effects observed with e^6MP. The addition of excess hypoxanthine and adenine also did not prevent growth inhibition by e^6MP (data not presented). These findings are contrary to those observed when the nucleobase analogs are substrates for purine salvage. 6-Methylmercaptopurine ribonucleoside induces differentiation of HL-60 cells via salvage by adenosine kinase, and adenine can block this differentiation [20]. Likewise, the HGPRT-mediated salvage of 6-thio-3-deazaguanine leads to differentiation of HL-60 cells, and hypoxanthine inhibits this process [14]. These data suggest that the mechanisms involved in the induction of differentiation by nonsalvaged nucleobase analogs are distinct from those of analogs that are substrates for purine salvage.

IMP dehydrogenase inhibitors have been shown to induce differentiation of HL-60 cells by decreasing the intracellular guanine nucleotide pools [21]. We found no alteration in the GTP pool between control wild-type HL-60 cells and cells treated with e^6MP for 2 h (Fig. 6), a time during which significant changes in c-*myc* mRNA expression were observed (French et al., manuscript in preparation). Furthermore, no new peaks were observed in the HPLC chromatogram, verifying that e^6MP is not salvaged by wild-type HL-60 cells. Therefore, the mechanism by which e^6MP inhibits cell growth is not due to alterations in the guanine nucleotide pools or to cytotoxic nucleotides of e^6MP.

A novel mechanism for the involvement of TG in the induction of differentiation in HGPRT-deficient HL-60 cells has been demonstrated by Kretz et al. [11]. TG acts as a substrate for tRNA-guanine ribosyltransferase and is incorporated into the anticodons of tRNAs normally containing queuosine. The addition of queuine, even at an 800-fold lower concentration, blocks TG-induced differentiation. However, the mechanism of e^6MP growth inhibition appears to differ from that of TG. Radiolabelled dihydroqueuine incorporation into the anticodon of tRNA was not inhibited by e^6MP (Fig. 7). Analysis of histidine tRNA, a queuosine-containing tRNA species, by RPC-5 chromatography also showed no change in the isoacceptor profiles, confirming that inhibition of queuine incorporation is not the mechanism by which e^6MP functions (data not presented).

Further studies are under way to elucidate the mode of action by which e^6MP elicits its effects on HL-60 cells. Although e^6MP is not an inhibitor of queuine incorporation into specific tRNA isoacceptors, other tRNA isoacceptors have highly modified nucleobases in their anticodon loops which could be affected. The possibility exists that a mechanism analogous to that of TG exists, except that other tRNA isoacceptors may be involved. Regardless of the ultimate mode of action, e^6MP might offer unique therapeutic potential based on its ability to inhibit the growth of wild-type and HGPRT-deficient HL-60 cells without inducing the usual cytotoxicity associated with purine analogs. This capability is not shared by most other purine analogs evaluated.

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