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Induction of HL-60 leukemia cell differentiation by tetrahydrofolate inhibitors of de novo purine nucleotide biosynthesis*

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Summary. 5,10-Dideazatetrahydrofolic acid (DDATHF) is a folate antimetabolite that shows activity against glycinamide ribonucleotide (GAR) transformylase, a folate-requiring enzyme in the de novo purine nucleotide biosynthetic pathway. Previous studies from our laboratory have shown that DDATHF is an effective inducer of the maturation of HL-60 promyelocytic leukemia. In solution, DDATHF is a mixture of two diastereomers due to an asymmetric configuration at carbon 6. Incubation of HL-60 cells with 1 μM of each diastereomer resulted in an inhibition of cellular proliferation after 48 h that preceded an increase in the number of differentiated myeloid cells, as determined by the ability of cells to reduce nitroblue tetrazolium (NBT) and by the binding of the myeloid-specific antibody Mo 1. Several analogs of DDATHF were also tested as inducers of the differentiation of HL-60 cells. With the exception of the 10-acetyl analog of 5-deazatetrahydrofolic acid, all compounds displayed similar activities as inducers of maturation. The finding that both stereoisomers of DDATHF, as well as the analogs tested, could selectively reduce intracellular purine nucleotide levels suggested that these compounds inhibited purine nucleotide biosynthesis de novo. This possibility was confirmed by the finding that hypoxanthine completely prevented the reduction of intracellular purine nucleotide levels, as well as the induction of differentiation and the inhibition of cellular growth, by these folate analogs. The results suggest that GAR transformylase is a target for a series of compounds whose structures resemble that of tetrahydrofolate and indicate that the inhibition of GAR transformylase by these compounds is sufficient to induce the maturation of HL-60 leukemia cells.

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Abbreviations: DHFR, dihydrofolate reductase; 5-DATHF, 5-deazatetrahydrofolic acid; DDATHF, 5,10-dideazatetrahydrofolic acid; GAR transformylase, glycinamide ribonucleotide transformylase; PBS, phosphate-buffered saline; NBT, nitroblue tetrazolium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AICA, 5(4)-amino-4(5)-imidazole carboxamide; FTIC, fluorescein isothiocyanate; DMSO, dimethylsulfoxide

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Introduction

Hematopoietic tumors represent the clonal expansion of transformed cells arrested at a particular stage of development [18]. Thus, the normal regulation of cellular proliferation and differentiation is altered in these malignant cells, resulting in a reduction in the probability of differentiation. Such imbalance leads to a marked amplification of the growth of immature leukemic cells [16, 20]. The HL-60 human leukemia cell line, which was originally derived from a patient with acute promyelocytic leukemia [9], undergoes continuous growth in culture yet retains the capacity to differentiate to mature granulocyte- or monocyte-like cells with restricted proliferative potential when exposed to a variety of chemical compounds, including several anticancer agents [6, 7, 10, 11, 18, 19]. Since the biochemical and morphological maturation of HL-60 cells in vitro resembles normal hematopoiesis, the development of therapeutic regimens of chemical inducers that increase the probability of completion of a terminal maturation program may offer an approach to the treatment of the leukemias.

The antifolates are important chemotherapeutic agents in the treatment of human cancer. The antitumor activity of antifolates is derived from the effective inhibition of dihydrofolate reductase (DHFR), which interferes with the recycling of oxidized folates to metabolically active reduced forms [8]. Thus, the cytotoxicity of antifolates is thought to be due to the reduction of intracellular levels of reduced folates, which are necessary for the biosynthesis of purine nucleotides and thymidylate. The observation that classic antifolates such as methotrexate can undergo polyglutama-

tion intracellularly, resulting in compounds with increased affinity for enzymatic sites other than that of DHFR, has led to the development of several novel antifolates that show primary activity against these targets [15, 25]. The antifolate 5,10-dideazatetrahydrofolic acid (DDATHF) displays little or no inhibitory activity against DHFR and thymidylate synthase [3]. Instead, DDATHF has been shown to be a specific inhibitor of glycinamide ribonucleotide (GAR) transformylase, the first of two enzymes in the de novo purine nucleotide biosynthetic pathway that are dependent on 10-formyltetrahydrofolate as a methyl donor, following the formation of DDATHF polyglutamates in a variety of cells [3]. The inhibition of GAR transformylase by DDATHF results in a marked depletion of intracellular purine nucleotide levels [4].

Initial studies have shown that DDATHF exhibits significant antitumor activity against a variety of murine solid tumors [3]. Since methotrexate, a classic antifolate that targets DHFR, displays little or no activity against these tumors, increased attention has recently been focused on the development of novel antifolates that show activity against targets of folate metabolism such as GAR transformylase. We have shown that DDATHF is an effective inducer of the maturation of HL-60 leukemia cells [23]. The induction of differentiation of HL-60 cells by DDATHF was preceded by a marked reduction in intracellular pools of purine nucleotides. The finding that the termination of cellular proliferation by DDATHF and the formation of a population of phenotypically mature cells by this agent were completely prevented by the simultaneous addition of exogenous purines and intermediates of purine nucleotide biosynthesis provided evidence that interference with the formation of purine nucleotides is an essential component of both biological events.

DDATHF in solution exists as two diastereomeric forms due to an asymmetric configuration at carbon 6 [3]. Both stereoisomers are substrates for mouse-liver folate polyglutamate synthase and are active in cell culture [3]. In this report, we extend our previous studies on the induction of the differentiation of HL-60 leukemia by DDATHF and demonstrate that each diastereomer of DDATHF has similar properties as an inducer of maturation of HL-60 cells. We also provide evidence that the capacity to induce HL-60 cellular maturation is common to a variety of synthetic tetrahydrofolates that resemble DDATHF.

Materials and methods

Chemicals. DDATHF and related compounds were kindly supplied by Dr. C. Shih, Eli Lilly Research Laboratories (Indianapolis, Ind.) Dimethylsulfoxide (DMSO), nitroblue tetrazolium (NBT), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and hypoxanthine were purchased from Sigma Chemical Co. (St. Louis, Mo.). The monoclonal antibodies Mo 1 and My 4 were obtained from Coulter Immunology (Hialeah, Fl.). All other chemicals obtained commercially were of reagent grade.

Cell culture. HL-60 promyelocytic leukemia cells were originally supplied by Dr. R. C. Gallo of the National Cancer Institute (Bethesda, Md.). The HL-60 cells employed in these experiments were expanded from stocks that had been frozen in liquid nitrogen, and all experiments used cells obtained between passage 27 and passage 65. Cell stocks were routinely checked for mycoplasma contamination using the gene-probe method (Gen-Probe, Inc., San Diego, Calif.).

Cells were passaged twice weekly in RPMI 1640 (Gibco, Grand Island, N. Y.) supplemented with 15% heat-inactivated (50° C for 30 min) fetal calf serum (Gibco). Cultures were maintained at 37° C in a humidified atmosphere containing 5% CO_2 in air. Experiments were performed by resuspending cultures of HL-60 cells at a density of 5×10^4 cells/ml in the presence of DDATHF or a related antifolate for 7 days. Solutions of DDATHF and synthetic folate derivatives were prepared in 0.1 *N* NaOH and adjusted to 10^{-3} M in phosphate-buffered saline (PBS) using an E_{273} nm (0.1 *N* NaOH) value of 9.15×10^{-5} M^{-1} cm^{-1}. Cell numbers were determined using a Coulter particle counter equipped with a channelizer (Coulter Electronics, Hialeah, Fl.).

Assessment of differentiation. The capacity of HL-60 cells exposed to DDATHF and other antifolates to undergo functional maturation was determined by measuring NBT dye reduction as previously described [22]. Approximately 1×10^6 cells were collected and resuspended in 1 ml RPMI 1640 containing 0.1% NBT and 1 μg/ml TPA (Sigma Chemical Co.). Stock solutions of TPA (1 mg/ml) were prepared in absolute ethanol and stored at –20° C. The cell suspension was incubated for 30 min at 37° C, and the percentage of cells containing blue-black formazan granules, indicative of a TPA-stimulatable respiratory burst, was determined by microscopic analysis, whereby a minimum of 200 cells were counted.

The phagocytic uptake of fluorescent microspheres (Fluoresbrite Carboxylate Microspheres; Polysciences, Inc., Warrington, Pa.) was measured as previously described [5]. Fluorescent microspheres were sterilized with 70% ethanol for 1 h, collected by centrifugation (1,500 g, 10 min, 4° C), and resuspended in RPMI 1640 containing 20% fetal bovine serum. HL-60 cells that had been exposed to antifolate for 7 days were washed and resuspended in fresh medium containing 3×10^6 fluorescent microspheres/ml. The concentration of microspheres was determined using a fluorescent microscope and a hemocytometer. Following incubation with microspheres for 16 h, cells were separated from free microspheres by centrifugation at 500 *g* for 5 min at 4° C, washed twice with ice-cold PBS, and analyzed immediately by flow cytometric analysis. Flow cytometry was performed with a Becton Dickinson fluorescent activated cell-sorter (FACS IV; Becton Dickinson, San Jose, Calif.) as previously described [5].

Analysis of ribonucleotide pools. HL-60 leukemia cells ($1-2 \times 10^5$ cells/ml) were suspended in fresh RPMI 1640 containing 1 μM DDATHF. At intervals, 2×10^6 cells were collected by centrifugation, washed twice in 2 ml ice-cold PBS, and extracted for analysis by HPLC as previously described [22].

Immunofluorescent staining of cell surfaces. The binding of the fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies Mo 1 and My 4 to the surface of HL-60 leukemia cells treated with DDATHF or related compounds was assessed by direct immunofluorescence using Coulter Clone monoclonal antibodies (Coulter Immunology, Hialeah, Fl.). The lyophilized FITC-labeled antibodies were prepared by reconstitution with distilled water followed by centrifugation at 100,000 *g* for 10 min as recommended by the manufacturer. Next, 1×10^6 HL-60 cells that had been exposed to DDATHF or a related derivative for the indicated interval were harvested and washed twice with PBS. FITC-labeled antibody binding to the cell population was quantified using a FACS analyzer (Becton Dickinson). The results, expressed as relative intensity, represented the mean fluorescence intensity of 20,000 cells as compared with that of cells incubated in the absence of FITC-labeled antibody. HL-60 cells treated with 1 μM retinoic acid, 1.5% DMSO, or 40 nM TPA were used as positive controls of differentiation. The presence of dead or damaged cells was determined by preincubating cultures with 2 μg/ml propidium iodide for 5 min prior to analysis [14]. In these experiments, <2% of the cells were stained with the propidium iodide; these cells were removed from the analysis by electronic gating.

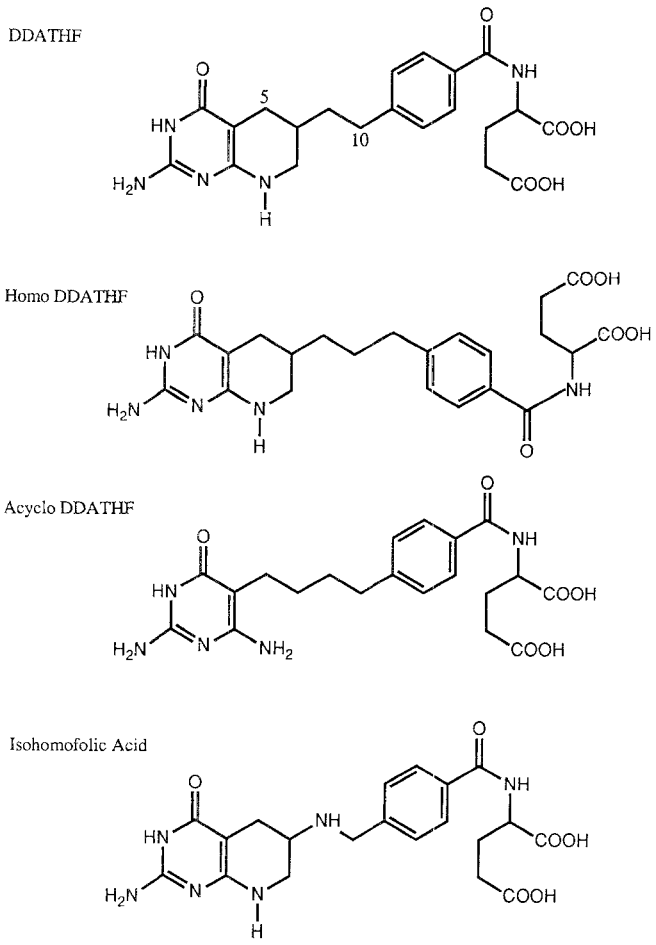

Fig. 1. Chemical structures of DDATHF analogs

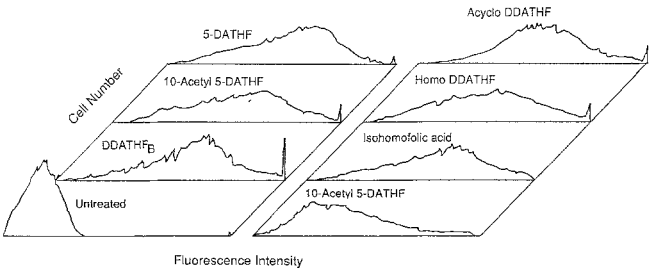

Fig. 2. FACS analysis of myeloid-specific antigens on HL-60 cells induced to differentiate by DDATHF and analogs. Cells were cultured in the presence or absence of 1 μM DDATHF and related compounds for 7 days. At that time, cells were removed and stained by direct immunofluorescence for the myeloid-specific antigen Mo 1 as described in Materials and methods

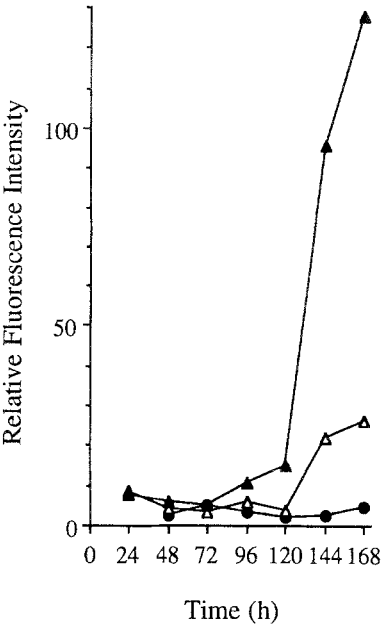

Fig. 3. Time course of the expression of the Mo 1 antigen on HL-60 leukemia cells induced to differentiate by DDATHF. Cells were cultured in the presence (▲) or absence (△) of 1 μM DDATHF for up to 7 days. Each day, aliquots of 2×10^6 cells were removed and stained by direct immunofluorescence with an anti-Mo 1 monoclonal antibody and then compared with cells prepared in the absence of Mo 1 antibody (●). Data derive from a single experiment representative of 3 separate experiments

Results

Exposure of HL-60 promyelocytic leukemia cells to DDATHF (see Fig. 1 for structure) has been shown to produce a concentration-dependent appearance of a population of cells expressing NBT positivity [23]. The Mo 1 antigen, a surface marker that increases with the granulocytic or monocytic maturation of HL-60 cells [12, 26], was also assessed by monitoring the binding of FITC-labeled Mo 1 antibody to cells that had been treated with these compounds. Whereas undifferentiated HL-60 promyelocytes expressed low levels of Mo 1 antigen (Fig. 2), HL-60 leukemia cells that had been treated with 1 μM DDATHF exhibited a time-dependent increase in Mo 1 levels that was apparent by 96 h, with maximal Mo 1 expression being observed on day 7 (Fig. 3).

The DDATHF used was a mixture of two diastereomers that result from the tetrahydro configuration at carbon 6. Exposure of HL-60 leukemia cells to 1 μM of each of the diastereomers for 7 days, the optimal concentration for induction of differentiation using the unresolved mixture, resulted in a level of differentiation by each diastereomer that was comparable with that produced by the unresolved mixture. Thus, as shown in Table 1, each diastereomer of DDATHF (DDATHF$_A$ and DDATHF$_B$) was equipotent as an inducer of HL-60 maturation, as measured by the formation of NBT-positive cells and the development of the capacity to phagocytize latex beads. This represented an increase in the number of differentiated cells and was not attributable to selective cytotoxicity to undifferentiated cells, since the yield of differentiated cells after exposure to DDATHF was 2- to 3-fold greater that present in the initial inoculum. In addition, HL-60 cells that had been exposed to each diastereomer of DDATHF for 7 days were shown to express high levels of the myeloid-specific antigen Mo 1. No binding of anti-Mo 1 monoclonal antibody to undifferentiated HL-60 promyelocytic leukemia cells was detected. Furthermore, <2% of the cells were positively stained by propidium iodide, a marker of cell membrane permeability [14], suggesting that the increase in Mo 1 binding was not the result of an increase in nonviable cells. Each diastereomer of DDATHF also caused a small increase in the expression of the monocytic specific antigen My 4 (Fig. 2, Table 1).

Table 1. Effects of antifolates on the growth and differentiation of HL-60 leukemia cells

Compound	Cells ($\times 10^{-5}$)	% NBT-positive cells	% Phagocytic cells	% Positive cells	
				Mo 1	My 4
None	18.5±1.3	2.5±0.3	4 ±0.6	5.3±0.2	5.2
$DDATHF_A$	4.1±0.3	70 ±4.3	33.2±0.1	52.8±2.7	11.2
$DDATHF_B$	4.4±0.4	72.4±4.9	37.5±2.3	67.1±9.4	12.6
5-DATHF	4.3±0.3	62.5±4.4	36.7±1.1	66 ±8.5	19.9
10-Acetyl-5-DATHF	5.6±0.6	25.4±4.9	21.1±2.2	45.6±9.2	3.3
10-Formyl-5-DATHF	4 ±0.1	64 ±6.7	39.8±1.2	59.1±15.5	12.9
Isohomofolic acid	4.1±0.2	64.4±3.4	40.4±3.2	65.4±7.3	10.4
Homo DDATHF	4.1±0.2	67.2±3.5	41 ±0.7	52.9±7.6	8.5
Acyclo DDATHF	4.2±0.2	73.5±2	41.6±2	67.5±3.5	11.8
DMSO	4.2±0.1	91.0	49.9	ND	ND

HL-60 leukemia cells were exposed to 1 μM of each of the antifolates. Cellular growth rates and the induction of differentiation were assessed on day 7 as described in Materials and methods. Data represent the means ± SE of at least 3 separate determinations, with the exception of those on DMSO (1.3%), which (except for growth) represent the mean of 2 determinations, and those on the percentage of cells positive for the My 4 antibody, which were taken from an experiment representative of 2 separate experiments. ND, Not determined

Table 2. Effects of hypoxanthine on the inhibition of the growth and differentiation of HL-60 leukemia cells by antifolates

Compound	–Hypoxanthine		+Hypoxanthine	
	% Control growth	% NBT-positive cells	% Control growth	% NBT-positive cells
Control	100	2 ±0.3	105.2±1.9	1 ±0.5
$DDATHF_A$	21.6±3.3	74 ±3.7	95.9±2.7	2.6±1
$DDATHF_B$	21.7±3.3	76.5±3.3	105.7±5.3	3.6±0.8
5-DATHF	23.2±4.5	77.8±1.5	100.7±2.5	4.4±1.5
10-Acetyl-5-DATHF	36.7±11.5	17.3±0.9	102.9±1.7	4 ±1
10-Formyl-5-DATHF	23 ±4.1	71.8±1.8	97 ±3.4	0.3±0.3
Isohomofolic acid	22.5±3.9	76.8±1.6	100.9±1.6	1.3±0.9
Homo DDATHF	25 ±4.2	67.1±1.6	104.7±2.4	0.9±1
Acyclo DDATHF	23.7±3.7	78.6±2.8	95.2±2.1	1 ±0.6

HL-60 leukemia cells were incubated with 1 μM of each derivative in the presence or absence of 100 μM hypoxanthine. Cell numbers and NBT positivity were measured on day 7. Data represent the means ± SE of at least 3 separate experiments using each compound

A series of tetrahydrofolic acid derivatives (Fig. 1) that resemble DDATHF by virtue of the replacement of one or both of the nitrogen atoms in the 5- and 10-positions of the molecule have recently been synthesized. Of this series of agents, the 10-acetyl derivative of 5′-deazatetrahydrofolic acid (10-acetyl-DATHF) was the least effective as an inducer of maturation of HL-60 cells (Table 1). Interestingly, the potent differentiation-inducing activity noted for both sterioisomers of DDATHF was also observed for derivatives that contain an extended propyl side chain linking the benzoylglutamate moiety to the 2-amino-4-oxopyrimidine ring, as well as for an acyclo derivative of DDATHF.

The finding that the induction of differentiation and the loss of cellular proliferative capacity by each of these compounds was completely prevented by simultaneous exposure to the purine nucleotide precursor hypoxanthine (Table 2) provides evidence that depletion of purine nucleotide pools may be a common feature of the mechanism of action of each of these compounds as well as of the resolved diastereomers of DDATHF. This was confirmed by studies demonstrating that each of the diastereomers of DDATHF, as well as the newly synthesized analogs, caused a pronounced reduction in the intracellular levels of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) following incubation with HL-60 leukemia cells in the absence of a source of hypoxanthine in the incubation medium (Table 3). Uridine triphosphate (UTP) levels were markedly increased under these conditions. Treatment of HL-60 leukemia cells with each of these compounds in the presence of 100 μM hypoxanthine completely prevented the reductions in purine nucleotide pools produced by the antifolates (Table 3).

Discussion

Treatment of HL-60 promyelocytic leukemia cells with DDATHF results in a reduction in cellular growth by 72 h; this event is preceded by a diminution of intracellular purine nucleotide levels, which in turn appears to be responsible for the induction of phenotypic changes characteristic of a differentiated phenotype [23]. The induction of HL-60 maturation and subsequent inhibition of cellular growth by DDATHF is completely prevented by simultaneous exposure of cells to hypoxanthine or 5(4)-amino-4(5)-imidazolecarboxamide, implying a role for intracellular purine nucleotide pools in the mechanism by which maturation is induced by this agent [23].

Table 3. Effects of hypoxanthine on nucleoside triphopsphate levels of HL-60 leukemia cells exposed to antifolates

Compound	% of Control					
	–Hypoxanthine			+Hypoxanthine		
	UTP	ATP	GTP	UTP	ATP	GTP
Control	100.0	100.0	100.0	40.0	85.9±1.3	89.4± 6.9
$DDATHF_A$	166.6±12.5	28.6±1.0	13.0±0.9	52.5	101.3±1.3	93.7± 4.7
$DDATHF_B$	156.1±35.8	30.6±4.4	21.9±3.2	48.5	92.2±9.6	99.8±19.1
5-DATHF	171.7±28.1	29.6±3.0	32.3±7.3	50.6	91.0±9.5	95.6±12.8
10-Acetyl-5-DATHF	156.8±36.6	59.9±2.7	63.3±9.7	49.9	94.5±5.5	99.7±10.7
10-Formyl-5-DATHF	159.2±24.5	27.9±1.7	31.0±3.8	ND	84.5	79.6
Isohomofolic acid	134.4±27.1	27.7±1.9	30.4±4.2	42.1	95.1±4.2	98.7± 4.2
Homo-DDATHF	140.8±20.8	27.7±1.9	30.4±4.2	41.7	91.3±2.4	93.2± 7.9
Acyclo-DDATHF	113.2±12.8	25.6±2.9	26.3±3.7	47.1	94.7±0.9	93.9± 5.7

HL-60 leukemia cells were exposed to antifolates in the presence or absence of 100 μM hypoxanthine for 24 h. Nucleoside triphosphate levels were analyzed as described in Materials and Methods. Average control nucleotide concentrations in nmol/10^6 cells were: UTP, 0.55; ATP, 2.16; GTP, 1.30. Data are the means ± S.E. of at least 3 experiments for each compound, with the exception of 10-formyl-DATHF and UTP plus hypoxanthine, which are the mean of 2 experiments. ND, not determined

In solution, however, DDATHF is a mixture of two diastereomers due to the tetrahydro configuration at the 6-position. In this report, we demonstrated that the resolved diastereomers of DDATHF exhibit similar activity as inducers of maturation of HL-60 cells at the concentration of the unresolved mixture that produces optimal induction of differentiation. We also showed that the capacity to induce HL-60 differentiation is a feature common to a series of derivatives of tetrahydrofolic acid that structurally resemble DDATHF. Thus, as inducers of maturation of HL-60 cells, each of these compounds except 10-acetyl-DATHF had properties essentially identical to those of DDATHF. The observation that each derivative caused a pronounced reduction in intracellular levels of ATP and GTP implies that the depletion of purine nucleotide pools is important in the mechanism of action of each of these compounds. This was confirmed by the finding that the simultaneous addition of hypoxanthine completely prevented the depletion of intracellular purine nucleotides by these antifolates as well as their capacity to affect cellular proliferation and induction of differentiation.

HL-60 promyelocytic leukemia has been used extensively as a model of hematopoietic development. The block in cellular differentiation can be reversed by a variety of chemical agents, including several compounds that diminish intracellular GTP levels. These include tiazofurin and related inhibitors of inosine 5′-phosphate dehydrogenase [22] as well as the de novo inhibitor of purine nucleotide biosynthesis, 6-methylmercaptopurine ribonucleoside [21], which causes the depletion of both GTP and ATP levels. The antifolate methotrexate is also an effective inducer of maturation of HL-60 cells due to its inhibition of purine nucleotide biosynthesis de novo [6]. However, the marked cytotoxicity of this agent complicates the study of its mechanism of action [24]. DDATHF was originally designed as an inhibitor of folate metabolism that affected an enzymatic target other than DHFR, the primary site of action of methotrexate. The recent observation that polyglutamate metabolites of methotrexate are effective inhibitors of several folate-dependent enzymes distal to DHFR, such as thymidylate synthase [1], 5(4)-aminoimidazole-4(5)-carboxamide transformylase [2], and GAR transformylase [17], suggested that novel antifolates could be developed that exhibit selective activity against these targets. DDATHF closely resembles tetrahydrofolate, a known inhibitor of GAR transformylase [13]. The presence of a fully reduced ring and of the 2-amino-4-oxo substituents rather than the 2,4-diamino pattern common to classic antifolates is believed to be responsible for the lack of inhibition of DHFR by DDATHF. Furthermore, the presence of carbon atoms instead of nitrogens at positions 5 and 10 completely prevents DDATHF from participating in one-carbon transfer reactions of folate metabolism and also provides its increased chemical stability. The similar level of differentiation-inducing activity exhibited by each of the antifolates used in this investigation as well as the resolved stereoisomers of DDATHF suggests that these compounds are excellent substrates for folylpolyglutamate synthase, thereby providing sufficient levels of the active metabolite to promote adequate depletion of intracellular purine nucleotide pools for the initation of the differentiation process.

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