Enhancement of the Differentiation-inducing Properties of 6-Thioguanine by Hypoxanthine and Its Nucleosides in HL-60 Promyelocytic Leukemia Cells

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ABSTRACT

Previous work has shown that 6-thioguanine (TGua) is an effective inducer of differentiation of Friend and HL-60 leukemia cells which lack hypoxanthine-guanine phosphoribosyltransferase but is at best only weakly active in inducing maturation in parental wild-type cells. Studies in wild-type and mutant HL-60 cells have provided evidence that the free-base TGua is the form of this drug that induces differentiation, while the formation of TGua nucleotides leads to cytotoxicity and inhibits differentiation. To attempt to increase the potential of TGua to serve as an inducer of parental HL-60 leukemia cells, physiological purine and pyrimidine nucleosides were tested for their ability to protect against TGua-induced cytotoxicity. Adenosine, deoxyadenosine, inosine, and deoxyinosine completely prevented the toxic action of the purinethiol, while guanosine and deoxyguanosine were only partially effective. The capacity of adenosine and deoxyadenosine to prevent the cytotoxicity of TGua was abolished by the inhibitor of adenosine deaminase, deoxyadenosine, implying that inosine and deoxyinosine were the active forms of the protecting agents. The protective activities of inosine and deoxyinosine appeared to depend on phosphorylation catalyzed by purine nucleoside phosphorylase, since exogenously added hypoxanthine was as effective as inosine in reducing the cytotoxicity of the parental antimitabolite. Accumulation of TGua nucleotides in the acid-soluble fraction of HL-60 cells treated with TGua was significantly decreased by the presence of inosine. Inosine also served under these circumstances as a d-ribose 1-phosphate donor to TGua, as evidenced by its increased conversion to 6-thioguanosine. Prevention of the cytotoxicity of TGua by the simultaneous administration of hypoxanthine or its nucleosides resulted in an expression of the antimitabolite's structure of TGua such that it does not serve as a substrate for the induction process. These findings support the concept that the processes of cytotoxicity and differentiation are separable events produced by different metabolic forms of the purine antimitabolite.

INTRODUCTION

A variety of compounds, such as cryoprotective agents (8, 31), hormones (24), vitamins (1, 4, 35, 37), tumor promoters (15, 25), and cancer-chemotherapeutic agents (34, 38), promote the differentiation of various cell types in culture. The inducers of maturation have chemical structures and biological activities that are so diverse that no unifiable concept on the underlying mechanism of induction has been reached. Among the chemotherapeutic agents, several antimetabolites such as 3-deazauridine, pyrazofurin (3), mycophenolic acid (26), and xylosyladenine (10) have been reported to be potent inducers of the differentiation of the Friend murine erythroleukemia and the HL-60 human promyelocytic leukemia. The optimum concentrations of these analogues for the induction of maturation are in the range where they elicit cytotoxicity, suggesting that the mechanisms responsible for both processes are identical. This concept is supported by the finding that xylosyladenine (10) and bromodeoxyuridine (20) are devoid of inducing ability in mutant cell lines with deletions in adenosine kinase and thymidine kinase, respectively. Furthermore, induction of the erythroid differentiation of Friend cells by the aminonucleoside of puromycin is inhibited by Ino3 (21), an antagonist of this analogue nucleoside (36). These results are collectively supportive of the concept that the activation of these analogues to the nucleotide level is essential for the induction process.

TGua is an exception to these findings, in that this purine antimitabolite is a potent inducer of differentiation of both Friend (13, 33) and HL-60 (9, 18) cells which lack HGPRT activity, but it is at best only weakly active as an initiator of maturation in wild-type parental cells (9, 18, 29). Analysis of intracellular and extracellular metabolites of TGua in HGPRT-deficient HL-60 cells exposed to this agent demonstrated negligible metabolism of TGua, a finding which supported the conclusion that the free-base TGua is the active form that induces differentiation, while in parental HL-60 cells, formation of TGMP leads to the generation of cytotoxicity (18). These results demonstrate that the metabolic forms of this 6-thiopurine that induce differentiation and exert cytotoxicity are different, indicating that these are separable events.

Several approaches appear to be possible to overcome the inability of TGua to function as an effective inducer of differentiation in wild-type HL-60 cells; these include (a) modification of the structure of TGua such that it does not serve as a substrate for HGPRT but remains at the base level, and (b) combination of TGua with other agents that reduce the formation of TGMP. In this paper combinations of TGua with Hyp and its nucleosides were used to effectively induce differentiation of parental HL-60 leukemia cells. This action appears to be due to the prevention of the formation of TGMP from TGua by the physiological purine and its nucleosides.

MATERIALS AND METHODS

HL-60 human promyelocytic leukemia cells were provided by Dr. Robert C. Gallo of the National Cancer Institute. The maintenance of this cell line and its derivatives has been described in detail previously (33). The abbreviations used are: Ino, inosine; TGua, 6-thioguanine; TGuo, 6-thioguanosine; TGMP, 6-thioguanosine 5'-phosphate; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; Ado, adenosine; dAdo, deoxyadenosine; Guo, guanosine; dGuo, deoxyguanosine; dino, deoxyinosine; Cyd, cytidine; dCyd, deoxycytidine; Urd, uridine; Thd, thymidine; PRPP, 5-phosphoribosyl 1-pyrophosphate; NBT, nitroblue tetrazolium; Hxp, hypoxanthine; dCF, deoxycytoformycin; Gu, guanine.

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2 To whom requests for reprints should be addressed.
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line and the isolation and characterization of the HGPRT− clone of HL-60 have been described previously (18). The percentage of inhibition of cellular growth was calculated based on the log of the cell number as described earlier (18). TGua and Hyp were dissolved in 0.1 and 0.5 N NaOH at concentrations of 50 and 250 μM, respectively; physiological nucleosides were dissolved in water. The extent of differentiation was assessed by measuring the functional capacity of individual cells to reduce NBT dye; the percentage of cells containing intracellular blue-black formazan deposits was measured using a hemocytometer under light microscopy (33). Analysis of extracellular metabolites of TGua was carried out by high-performance liquid chromatography as detailed previously (18), and measurement of the accumulation of TGua nucleotides in the acid-soluble fraction was quantitated by the fluorometric assay developed by Tidd and Dedhar (39). Briefly, parental HL-60 cells were treated with TGua in the presence or absence of Ino. Cells were then washed 2 times with growth medium and once with phosphate-buffered saline (0.2 g KCl-8 g NaCl-0.2 g KH2PO4-1.15 g Na2HPO4 per liter, pH 7.4) and extracted with 0.5 N perchloric acid. TGua nucleotides in the extracts were oxidized with alkaline-permanganate and subjected to fluorometry.

RESULTS

The growth-inhibitory activity of various physiological nucleosides against parental and HGPRT− HL-60 cells was measured, and the results are shown in Table 1. Ado and dAdo u.c.d in combination with dCF exerted the greatest cytotoxicity to both cell lines, which was comparable in magnitude. Guo and dGuo were 10 to 20 times less cytotoxic to parental HL-60 cells than the combination of Ado and dAdo with dCF. As expected, HGPRT− HL-60 were relatively resistant to Guo, while dGuo exerted cytotoxicity in this cell line, presumably due to phosphorylation catalyzed by deoxycytidine kinase (12). Ino, dino, and pyrimidine nucleosides, on the other hand, were in general considerably less toxic to both cell lines, with the dose required for 50% inhibition of cellular replication being more than 4 mM. Thd was an exception, in that it was relatively cytotoxic to both cell lines.

The effects of these nucleosides on the growth-inhibitory activity of TGua against both parental and HGPRT− cells are shown in Table 2. The levels of TGua to which parental and HGPRT− HL-60 cells were exposed were 8 μM and 0.5 mM, respectively; at these concentrations, the growth of each cell line was inhibited by 50%. Nucleosides were evaluated by measuring the change in the cytotoxicity of TGua produced by the continuous exposure of cells simultaneously to the purinethiol and the nucleoside under test, with toxic nucleosides being used at a level that had minimum effects on cell proliferation, and relatively nontoxic nucleosides given at a concentration of 1 mM. None of the nucleosides used decreased the toxicity of TGua to HGPRT− HL-60 cells, and slight enhancement of the growth inhibition produced by the purine antimetabolite in this cell line occurred with Ino. In contrast, Ado, dAdo, Ino and dino completely prevented the cytotoxicity of the 6-thiopurine, and Guo and dGuo were partially protective, in parental HL-60 cells. In the presence of dCF, Ado and dAdo at levels of 10 to 25 μM were ineffective in preventing the toxicity of TGua to parental HL-60 cells (data not shown). These findings suggested that the protective effects of Ado and dAdo were attributable to deamination to Ino and dino, respectively. However, since Ino and dino can be degraded intracellularly to Hyp by purine nucleoside phosphorylase, it appeared that the protective effects of Ino and dino might well be due to their conversion to Hyp. To test this possibility, the effects of Hyp on the cytotoxicity of TGua were measured (Table 3). The 50% inhibitory values for TGua in the presence of Hyp, Ino and dino were comparable, except at the highest concentra-

<table>
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<tr>
<th>Addition</th>
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<tbody>
<tr>
<td>0.01 mM</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
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<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>5</td>
</tr>
<tr>
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<td>55</td>
<td>21</td>
</tr>
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<td>24</td>
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<tr>
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<td>57</td>
<td>49</td>
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</table>

a ID50, dose required for 50% inhibition of cellular replication.
b dCIF added at 25 μM.
c ND, not determined.
Table 3
Comparison of the protective effects of Hyp, Ino, and dino on the cytotoxicity of TGua in parental HL-60 cells

<table>
<thead>
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<th>Concentration of protecting agent (mM)</th>
<th>Cytotoxicity (ID50 μM) for TGua</th>
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<td></td>
<td>Hyp</td>
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<tr>
<td>0.05</td>
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<tr>
<td>0.5</td>
<td>190</td>
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<tr>
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<td>280</td>
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</table>

* ID50, dose required for 50% inhibition of cellular replication.
* Data derived from Chart 1.

TGua causes differentiation of wild-type HL-60 cells to only a slight degree at a concentration of 0.5 μM, implying that the cytotoxicity of this agent predominates over its capacity to induce differentiation (18, 29). Conversely, due to prevention of the formation of the cytotoxic metabolite TGMP, the induction of differentiation is the predominant action in the termination of proliferation by TGua in HGPRT- HL-60 cells (18, 32); thus, the extent of differentiation in this cell line, as assessed by NBT dye reduction, a functional marker of mature phagocytic cells, was significant at levels of TGua at 0.4 to 0.7 mM (Chart 1d). Previous findings from our laboratory (18, 32) have demonstrated that the processes of cytotoxicity and the induction of differentiation by TGua are separate phenomena, with TGMP being the molecular form required to generate cytotoxicity and the free-base TGua having the capacity to induce differentiation. We, therefore, ascertained whether the lessening of the cytotoxicity of TGua in parental HL-60 cells by simultaneous exposure to Hyp, Ino, or dino permitted the expression of the differentiation-inducing properties of the 6-thiopurine. As shown in Chart 1, a to c, the simultaneous use of TGua with 2 mM hypoxanthine or its physiological nucleosides resulted in a marked decrease in the cytotoxicity of the purine antimetabolite, increasing the 50% inhibitory concentration of TGua from 8 μM when used alone to 0.23, 0.18, and 0.29 mM when the 6-thiopurine was combined with Ino, dino, or Hyp, respectively. Under these latter conditions, extensive differentiation occurred, which predominated over cytotoxicity at the lower drug concentrations with Ino and Hyp. Neither Ino, dino, nor Hyp at levels up to 2 mM was capable of inducing the maturation of parental HL-60 cells.

To gain information on the biochemical basis for the action of the physiological nucleosides in combination with TGua, the levels of intracellular TGua nucleotides were measured after the exposure of parental HL-60 cells to TGua in the presence and absence of Ino. Whereas accumulation of TGua nucleotides occurred in parental HL-60 cells treated with TGua alone, the presence of Ino effectively prevented the formation of TGua nucleotides over a wide range of concentrations of TGua (Chart 2). In addition, Ino served as a donor of α-ribose 1-phosphate and increased the conversion of TGua and TGuo. Thus, TGuo excreted into the medium of cultures treated with TGua and Ino constituted 32% of the total 340-nm UV-absorbing material, compared to only 6% in cells treated with TGua alone (Chart 3).
DISCUSSION

Previous studies from our laboratory (18) have provided evidence that TGua itself is the form of this antimitabolite that induces terminal differentiation of HGPRT- HL-60 leukemia cells. In parental HL-60 cells, the 6-thiopurine is at best a poor inducer of differentiation, presumably because TGua is extensively converted to TGMP, and cytotoxicity is the predominant expression of the action of this compound in wild-type cells (18, 29). These findings have demonstrated that different molecular forms of this antimitabolite are responsible for the expression of cytotoxicity and the initiation of maturation, separating these actions of the drug as distinct phenomena. In further support of this premise, termination of the proliferation of parental HL-60 cells as an expression of TGua-induced cytotoxicity is accompanied by accumulation of cells in the S and G2-M phases of cell cycle, while the production of end-stage HGPRT- HL-60 cells due to the differentiation produced by the purine antimitabolite causes cells to accumulate in G0 (32).

Other investigators have demonstrated that the cytotoxicity of antimitabolites, such as 5-fluorouracil, can be prevented by the presence of Thd (26, 41) or enhanced or decreased by Ino or dino, depending upon the cell type (2, 6, 40). Furthermore, the antitumor activity of 5-fluorouracil in vivo has been reported to be increased by the coadministration of Guo to tumor-bearing animals (16). Findings such as these encouraged the search for physiological nucleosides that were capable of selectively modulating the cytotoxicity of TGua, thereby permitting predominant expression of the differentiation-inducing properties of this agent.

Ino and dino were found to be capable of completely preventing the TGua-induced cytotoxicity to parental HL-60 cells. Prevention of TGua toxicity by Ado and dAdo also occurred but appeared to be attributable to the enzymatic deamination of these nucleosides to Ino and dino, respectively, because Ado and dAdo were devoid of protective activity when used in the presence of the inhibitor of adenosine deaminase, dCF. Furthermore, since Hyp was as effective as Ino and was superior to dino in preventing the cytotoxicity to TGua, it appeared that the effects of these nucleosides were due to their conversion to Hyp, through degradation by purine nucleoside phosphorylase.

Hyp can be visualized to protect against the cytotoxicity of TGua by preventing the anabolism of the purinethiol to its nucleotide form by exerting effects at several different levels. These include competition between Hyp and TGua for (a) the transport carrier, since the uptake of TGua is mediated by a Hyp-Gua carrier (30), (b) the enzyme HGPRT, the K_m values for Hyp and TGua with HGPRT from adenocarcinoma 755 being reported to be 4 and 10 μM, respectively (14), and (c) PRPP, with the activation of Hyp to IMP leading to a decreased availability of PRPP for the synthesis of TGMP by HGPRT. An alternate way by which Ino may circumvent the toxicity of TGua may be by stimulation of its conversion to TGuo, which should be relatively less toxic than TGua, since no kinase is known which effectively phosphorylates TGuo (22). None of these mechanisms is mutually exclusive. An earlier study, using labeled pyrimidine bases, has shown that Ino or dino can serve as a donor of d-ribose 1-phosphate or 2-deoxyribose 1-phosphate, respectively (11). This appears to also occur with the combination of TGua and Ino, as evidenced by the relatively great excretion of TGuo into the medium by cells treated with the combination of TGua and Ino. Thus, both Hyp and d-ribose 1-phosphate or 2-deoxyribose 1-phosphate, the products that result from phosphorylation of Ino or dino, may all contribute to the protective activities against TGua cytotoxicity exerted by these nucleosides. However, the finding that Hyp is as potent as Ino in exerting protection from the cytotoxicity of TGua would argue that the formation of TGuo is not a critical event. This is further supported by the results obtained with dino, a weaker substrate for purine nucleoside phosphorylase than Ino (19), which was significantly less potent in protecting cells from the cytotoxicity of TGua than Ino.

It is also conceivable that the antagonistic action of Hyp and its nucleosides may involve reversal of damage caused by the cytotoxic analogue. There is evidence that reversal of metabolic damage contributes to the prevention of 5-fluorouracil toxicity by Thd (27, 41) and the prevention of dGuo toxicity in purine nucleoside phosphorylase-deficient lymphoma cells by dCyd (12). TGua toxicity can also be partially reversed in HL-60 cells by the addition of Guo after a short period of treatment with TGua. In addition, Hyp has been shown recently to antagonize the cytotoxicity caused by 6-diazo-5-oxo-L-norleucine and 6-methylmercaptopurine in addition to TGua and 6-mercaptopurine (42), suggesting that a portion of the damage produced by TGua is reversible. TGua toxicity can also be partially prevented by the presence of inhibitors of DNA synthesis (7, 23, 28). The marked decrease in the intracellular accumulation of TGua nucleotides observed with the combined simultaneous treatment of HL-60 cells with TGua and Ino, however, indicates that prevention of cytotoxicity is being achieved by interference with the activation of the antimitabolite to its cytotoxic form(s). These results are compatible with the findings that a high degree of resistance to 6-purinethiols can be attained by a loss of HGPRT activity (5) or by a constitutive decrease in the intracellular pool of PRPP (17).

In an earlier report (18), 50% NBT-positive parental HL-60 cells were produced by treatment with 2 mM Hyp. In the current study, 2 mM Hyp produced less than 3% NBT-positive cells, and significantly higher concentrations of Hyp were required to initiate maturation in HL-60 cells. Similarly, responsiveness of HGPRT- HL-60 cells to the induction of differentiation by TGua was found to be slightly higher than in the previous study (18). In addition, a decrease in the percentage of cells of both parental and HGPRT- HL-60 cells undergoing spontaneous maturation has occurred. Thus, a gradual loss of responsiveness to natural and chemical inducers has occurred during passage generations 30 and 80, indicating the desirability of using early passage levels of HL-60 cells in studies of this kind.

The dose of TGua required to cause the differentiation of parental HL-60 cells in the presence of Ino or Hyp ranged from 0.1 to 0.2 mM, concentrations significantly lower than those required to induce the maturation of HGPRT- HL-60 cells. Since Hyp possesses inducing potential (13, 18), this phenomenon could be due to a significant degree of enhancement of the maturation process by the combination of inducers, even at concentrations which by themselves are ineffective as has been demonstrated previously (35). The ability of Hyp and its nucleosides to prevent the cytotoxicity of TGua and to allow the expression of the differentiation-inducing ability of the 6-thiopurine supports the concept that these processes are separable actions of the 6-thiopurine and suggests that the combination of TGua with Ino...
or Hyp might well be useful in the clinical use of the thiopurine as an inducer of maturation in the therapy of the acute leukemias.

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