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Induction of erythroid differentiation and modulation of gene expression by tiazofurin in K-562 leukemia cells

(IMP dehydrogenase/GTP/c-Ki-*ras* expression/embryonic hemoglobin)

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ABSTRACT **Tiazofurin (2-β-D-ribofuranosyl-4-thiazolecarboxamide; NSC 286193), an antitumor carbon-linked nucleoside that inhibits IMP dehydrogenase (IMP:NAD$^+$ oxidoreductase; EC 1.1.1.205) and depletes guanylate levels, can activate the erythroid differentiation program of K-562 human leukemia cells. Tiazofurin-mediated cell differentiation is a multistep process. The inducer initiates early (<6 hr) metabolic changes that precede commitment to differentiation; among these early changes are decreases in IMP dehydrogenase activity and in GTP concentration, as well as alterations in the expression of certain protooncogenes (c-Ki-*ras*). K-562 cells do express commitment—i.e., cells exhibit differentiation without tiazofurin. Guanosine was effective in preventing the action of tiazofurin, thus providing evidence that the guanine nucleotides are critically involved in tiazofurin-initiated differentiation. Activation of transcription of the erythroid-specific gene that encodes $^A\gamma$-globin is a late (48 hr) but striking effect of tiazofurin. Down-regulation of the c-*ras* gene appears to be part of the complex process associated with tiazofurin-induced erythroid differentiation and relates to the perturbations of GTP metabolism.**

Cancer can be viewed as a disorder generating from an uncoupling of gene expression that controls cellular proliferation and differentiation. Therefore, much attention has been paid to cancer cell lines that can be induced to differentiate after *in vitro* exposure to chemical or chemotherapeutic agents (for reviews, see refs. 1–3). The K-562 human leukemia cell line provides a useful system for studying human erythroid differentiation because it expresses markers of erythroid lineages, such as hemoglobin.

Of the variety of chemotherapeutic agents used in cancer treatment, some can induce cell differentiation (4). In our previous studies we used drugs directed against key enzymes of purine and pyrimidine metabolism (5–7). Activities of target enzymes of these drugs were those that were tightly linked with *in vivo* and *in vitro* proliferative activity (5, 8, 9). The activity of IMP dehydrogenase (IMP:NAD$^+$ oxidoreductase; EC 1.1.1.205), the rate-limiting enzyme of *de novo* GTP biosynthesis, markedly increased in various types of cancer cells; therefore, this enzyme was suggested as a sensitive target for anticancer chemotherapy (5, 10).

Tiazofurin (2-β-D-ribofuranosyl-4-thiazolecarboxamide; NSC-286193) potently inhibits the proliferation of a variety of experimental and human neoplasms and is now in phase I and II clinical trial (11, 12). Tiazofurin in sensitive cells is converted to 4-thiazolecarboxamide adenine dinucleotide, an NAD analogue. This active metabolite profoundly inhibits IMP dehydrogenase activity and depletes the metabolites of the guanylate biosynthetic pathway (7, 13–16). Recently inhibitors of IMP dehydrogenase were reported to induce differentiation *in vitro* in HL-60 promyelocytic leukemia cells (17, 18) and *in vivo* in patients with refractory acute myelogenous leukemia (12).

Guanine nucleotides are required for several metabolic pathways and functions of cancer cells (5). The G protein family was reported to represent a class of regulatory proteins (19). DNA sequence analysis revealed a significant similarity between one G protein subunit and the gene product of the *ras* protooncogene family (20). As with the G proteins, the normal c-*ras* proteins associated with plasma membrane bind GTP and GDP and have GTPase activity (21, 22).

In our study we demonstrate that tiazofurin induces erythroid differentiation in K-562 cells, causing a coordinated expression of inducer-initiated alterations. Tiazofurin initiates an early decrease in the activity of IMP dehydrogenase and in the intracellular concentration of GTP and causes down-regulation of c-Ki-*ras* gene. These changes occur before commitment to differentiation is accomplished, suggesting that these alterations may operate in regulating the tiazofurin-triggered differentiation program.

MATERIALS AND METHODS

Materials. Tissue culture medium was obtained from GIBCO. The [α-^{32}P]dATP (specific activity, ≈3000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Tiazofurin was provided by V. L. Narayanan (National Cancer Institute, Bethesda, MD).

Cell Lines and Cultures. K-562 cells were provided by E. Klein (Karolinska Institute, Stockholm). Cells were maintained in RPMI 1640 medium containing 10% fetal calf serum supplemented with penicillin (100 units per ml) and streptomycin (50 μg/ml) at 37°C in a humidified atmosphere with 5% CO_2. Cells at a concentration of 1×10^5 per ml were induced with 5 or 10 μM tiazofurin or with 45 μM hemin.

Hemoglobin Determination. Percentage of benzidine-positive cells was scored by counting 1000 cells. Benzidine stain was freshly prepared before use by adding 5 μl of 30% hydrogen peroxide to 1 ml of stock solution of 0.2% benzidine/0.5% acetic acid; 50 μl of this solution was used for 50 μl of cell suspension. Hemoglobin contents of cell lysates were determined from the visible absorption spectrum (23). Hemoglobins were separated by PAGE as described (24).

Enzyme Assay. Activity of IMP dehydrogenase was determined as reported (25).

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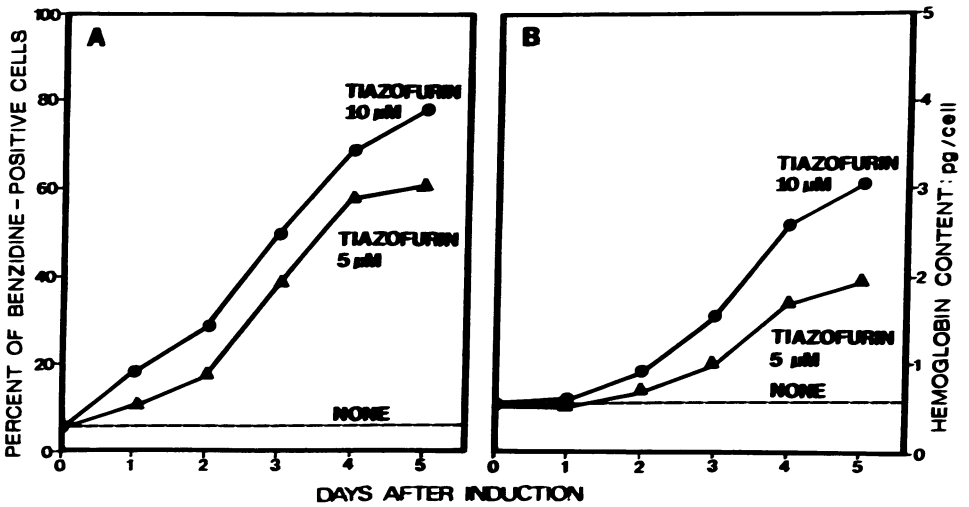

FIG. 1. Kinetics of hemoglobin induction in tiazofurin-treated K-562 cells. Percent of benzidine-positive cells (initial value = 5%) (*A*) and intracellular hemoglobin content (*B*) were determined as described (initial value = 0.5 pg per cell). Means of three experiments are given (SEM <5%). Cell viability was always >95% as monitored daily by the trypan blue dye exclusion test.

Clonogenic Assay. Each concentration of inducer was added to three 25-cm^2 tissue culture flasks containing 10 ml of complete medium and cells at 1×10^5 cells per ml. After 0, 12, 24, 48, and 72 hr, cells were washed with phosphate-buffered saline to remove inducer and then plated at 1500 cells per ml of RPMI 1640 medium/20% fetal calf serum/penicillin (100 units per ml)/streptomycin (50 μg/ml)/0.33% agar without inducer in a plastic 35-mm Petri dish. Five days later 1 ml of benzidine stain was added to each Petri dish, and 5–10 min later the colonies were scored under an inverted microscope at 100× magnification. Colonies were classified as only benzidine-positive, only benzidine-negative, or mixed.

Analysis of Ribonucleotide Pools. Cells were plated at 1×10^5 cells per ml of fresh complete medium containing the inducer of differentiation. After incubation for various periods, cells were harvested and extracted as described (8). Separation of nucleotides was done on a Pharmacia (Sweden) fast performance liquid chromatograph (FPLC) system equipped with an LCC-500 controller and by using a Pharmacia Mono Q HR 5/5 anion-exchange column (5 cm × 0.5 cm × 10 μm). An ammonium phosphate-buffer gradient (pH 7.0) ranging from 0.01 to 1.00 M at a flow rate of 1 ml/min was applied. UV absorbance of column eluates was detected with a UV-M monitor (Pharmacia) at 254 nm. Peak areas were measured using the integrator of LCC-500, and retention times were compared with external standards.

Molecular Probes. The human c-Ki-*ras*-specific plasmid PsW11-1 (26) containing a 1.1-kilobase (kb) *Pst* I–*Bam*HI fragment was obtained from R. A. Weinberg (Massachusetts Institute of Technology, Boston). A probe specific for $^A\gamma$-globin chain, provided by A. W. Nienhuis (National Cancer Institute), is a 2.5-kb *Hin*dIII fragment.

Assay for Gene Expression. Dot-blot hybridization analysis was done with total cytoplasmic RNA (27). After formaldehyde/SSC (1× SSC = 0.15 M sodium chloride/0.015 M trisodium citrate) denaturation (28), 10–1.25 μg of RNA was spotted onto nitrocellulose membranes, and the filters were hybridized to a nick-translated 1.1-kb *Pst* I–*Bam*HI fragment containing the human c-Ki-*ras* gene or to a nick-translated 2.5-kb *Hin*dIII–*Hin*dIII fragment carrying $^A\gamma$-globin-encoding gene, respectively. Protocol for the molecular hybridization consisted, first, of treatment with 50% (vol/vol) formamide/5× SSC at 42°C for 24 hr. Blots were then washed under stringent conditions—four times with 2× SSC containing 0.1% $NaDodSO_4$ at room temperature for 5 min and then twice with 0.1× SSC containing 0.1% $NaDodSO_4$ at 50°C for 15 min. Dot-blot filters were exposed on Kodak XAR-5 film at −70°C for 2–6 days with intensifying screen.

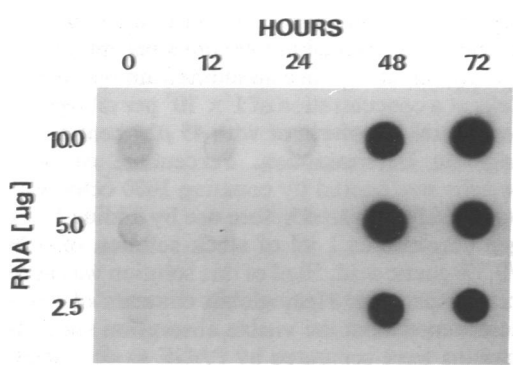

FIG. 2. Dot-blot analysis of IgA-encoding gene transcripts in total RNA prepared from K-562 cells at different periods of tiazofurin treatment. Total RNA (10, 5, or 2.5 μg) was dotted onto a nitrocellulose filter and then hybridized with ^{32}P-labeled probes as described.

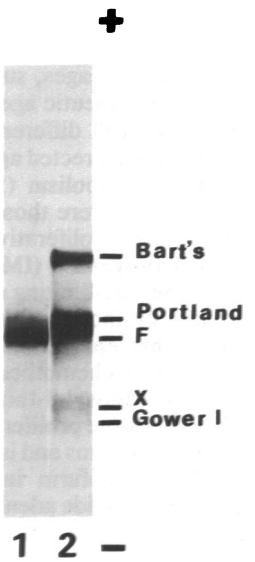

FIG. 3. Hemoglobin pattern in K-562 cell line after 96-hr exposure to tiazofurin. Lanes: 1, hemoglobin standards; 2, 10 μM tiazofurin.

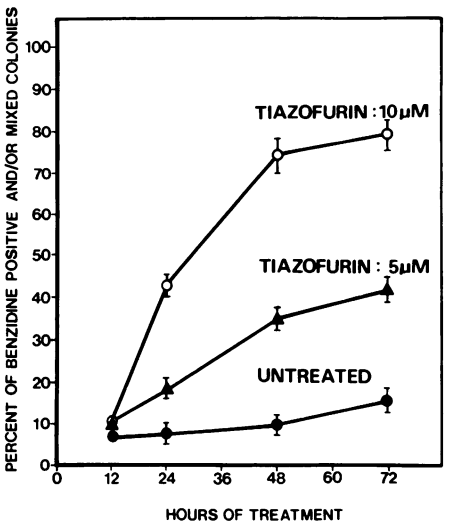

FIG. 4. Commitment assay. Cells were cultured for various periods with tiazofurin and then plated in semisolid medium without inducer. Five days later colonies were stained and scored for benzidine-positivity as described. There was a low spontaneous induction of hemoglobin synthesis of ≈5% during the 72-hr period. Means ± SEM of three or more experiments are shown.

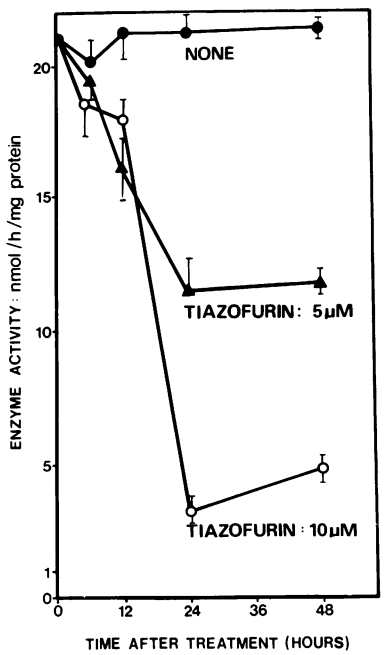

FIG. 5. Response of IMP dehydrogenase activity to tiazofurin treatment in K-562 leukemia cells. Cells were incubated with tiazofurin for various periods. The enzyme activity was measured in the cytosol as described (25). Means ± SEM of triplicate assays (or more) are given and plotted as percentages of corresponding control values. Specific activity of untreated cells at 0 hr was 21.0 ± 0.8 nmol/hr per mg of protein.

RESULTS AND DISCUSSION

Cell Proliferation and Hemoglobin Synthesis. Exponentially proliferating K-562 cells grew in culture with a doubling time of ≈24 hr. Exposure of cells to tiazofurin resulted in a dose-dependent decline of cell proliferation. On adding 5 μM or 10 μM tiazofurin, doubling time increased to ≈26 or 40 hr, respectively. After 5-day growth with 10 μM tiazofurin, the percentage of benzidine-positive cells increased to 78%, and an average of 3.1 pg of hemoglobin accumulated per cell (Fig. 1). Exposure of K-562 cells to 10 μM tiazofurin resulted in a marked elevation in the transcription of $^{A}\gamma$-globin-encoding gene. Globin-specific hybridization signals became more intense at 48-hr treatment (Fig. 2). Because tiazofurin was reported to cause differentiation in HL-60 human promyelocytic leukemia cells (17, 18), our work shows that this antimetabolite induced both erythroid and myeloid differentiation *in vitro*.

PAGE of hemoglobin from untreated K-562 cells showed that the hemoglobin mainly consisted of small amounts of embryonic forms: Hb Portland, Hb Gower I, and Hb F were detected (29). With tiazofurin, K-562 cells synthesized large amounts of Hb Portland, and bands corresponding to Hb Bart's, Hb X, and Hb F were very visible, but the Hb Gower I band resembled that of the control (Fig. 3). No Hb A bands were seen. A similar embryonic Hb pattern was detected

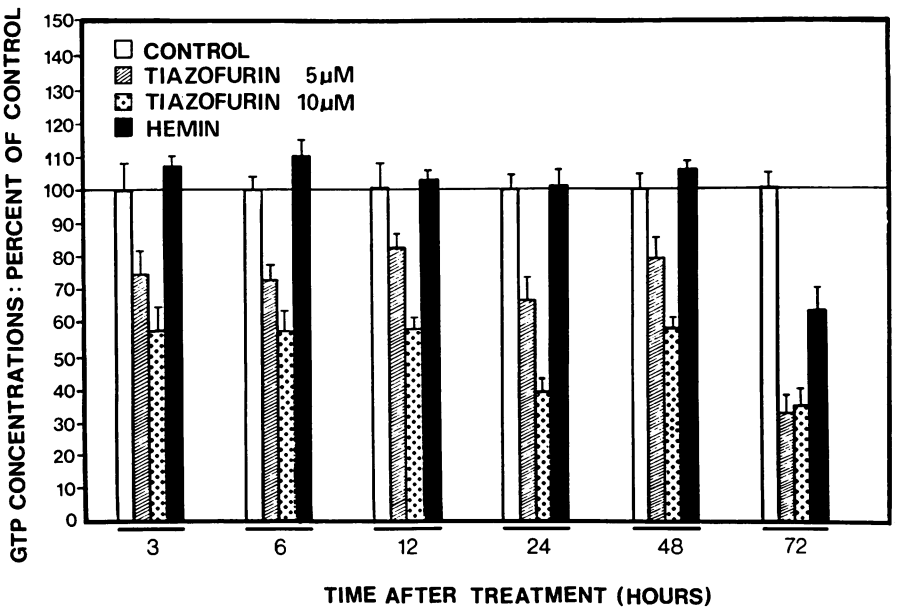

FIG. 6. Effect of tiazofurin on GTP concentration in K-562 human leukemia cells. K-562 cells (1×10^5 cells per ml) were exposed to 10 μM tiazofurin for various periods. Aliquots were analyzed by fast performance liquid chromatography as described. Means ± SEM of three or more samples are expressed as % control untreated cells. Average control nucleotide concentrations (in nmol per 10^6 cells) were as follows: ATP, 5.45; GTP, 1.77; UTP, 3.36; and CTP, 0.64.

during hemin-mediated erythroid differentiation of K-562 cells (29).

Commitment of K-562 Cells to Differentiation. To assess whether commitment occurs (i.e., the decision to express a differentiated phenotype without inducer), cells were cultured for various periods with tiazofurin and then plated in semisolid medium (containing 0.33% agar) without inducer. Five days after plating, colonies were stained with benzidine. Three types of colonies were found: those containing (*i*) only benzidine-negative cells, (*ii*) only benzidine-positive cells, and (*iii*) both cell types (mixed colonies). Negative colonies were large, whereas mixed colonies were generally of intermediate size. By contrast, benzidine-positive colonies were small, regardless of length of prior exposure to inducer, reflecting that differentiating cells exhibited restricted proliferation. With prior exposure to 10 μM tiazofurin, a 12-hr lag period existed before effects of the drug became irreversible (Fig. 4). Cells transferred to inducer-free medium after exposure to 10 μM tiazofurin for less than this lag period did not withdraw from the cell cycle, nor did they show any changes indicative of erythroid differentiation. Tiazofurin progressively increased the percentage of positive and mixed colonies.

Activity of IMP Dehydrogenase and GTP Concentrations During Induction by Tiazofurin. Previous studies showed that the metabolically active form of tiazofurin, 4-thiazolecarboxamide adenine dinucleotide, inhibits IMP dehydrogenase and thus depletes guanosine nucleotide pools (7, 13–16). Therefore, association of these events with expression of the differentiated phenotype was tested in K-562 cells. Fig. 5 shows that cell exposure to tiazofurin caused a drug-dose-dependent decrease in specific activity of IMP dehydrogenase. This decrease appeared by 6 hr of treatment with 10 μM tiazofurin and was most marked at 24 hr (15% of control values).

Concurrently, tiazofurin exposure rapidly depleted intracellular levels of GTP (Fig. 6). In contrast, concentrations of UTP and CTP increased at 6 hr (to 120–140% of control values); restoration to control levels of UTP and CTP was seen between 24 and 48 hr with a decrease by 72 hr. At this early time, ATP pools were not significantly altered (data not shown). Hemin, a known inducer of erythroid differentiation, did not reduce the GTP pool before 72 hr.

Guanosine Rescue. Because modulation of intracellular GTP level is an early measurable response to induction, determining whether depletion of GTP pools is critical to maturation was of interest. When cells were exposed concurrently to tiazofurin and guanosine, no increase of benzidine-positive cells occurred—i.e., guanosine could prevent tiazofurin action in K-562 cells (Table 1). Our results argue that the rapid drop in intracellular GTP concentration, seen when K-562 cells were treated with inducer, is a necessary event leading to differentiation rather than a by-product of induction. Our observation and conclusion are consistent with results gained in HL-60 cells with IMP dehydrogenase inhibitors (17, 18).

Expression of Protooncogenes. GTP has several functions and routes of utilization (5), including its ability to interact

Table 1. Effect of guanosine on the differentiation of K-562 cells

Treatment	Benzidine-positive cells, %
None (control)	7 ± 0.6
Tiazofurin (10 μM)	64 ± 5.8*
Tiazofurin (10 μM) + guanosine (50 μM)	53 ± 4.0*
Tiazofurin (10 μM) + guanosine (75 μM)	7 ± 0.5

Means ± SEM of three experiments are given.
*Significantly different from control ($P < 0.05$).

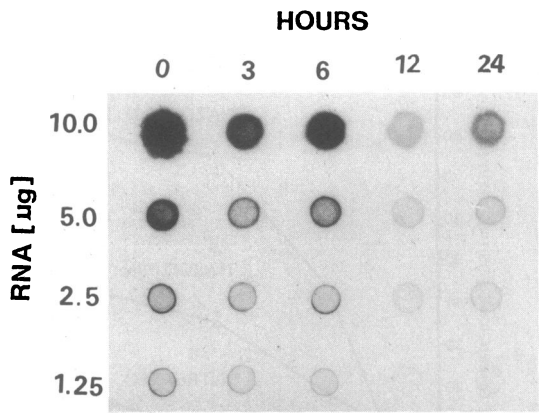

FIG. 7. Dot-blot analysis of c-Ki-*ras* protooncogene transcripts at different periods of tiazofurin treatment. Procedure was the same as that for Fig. 2.

with protein products of the c-*ras* oncogene family. The fact that, like the regulatory G proteins (19), the c-*ras* gene products associate with the plasma membrane and bind GTP suggested that the *ras* oncogene products act as GTP-dependent signal transducers that forward mitogenic signals between unidentified growth factors and effector systems (for review, see ref. 30). As proliferation and differentiation seem to be reciprocally linked processes, it was important to elucidate the tiazofurin-mediated modulation of expression of c-*ras* gene. Total cytoplasmic RNA that was extracted from K-562 cells at various times after induction was assayed by dot-blot analysis. In contrast to what is seen for other inducers of K-562 cells (hemin, sodium butyrate, 1-β-D-arabinofuranosylcytosine) (31, 32), one early molecular effect of tiazofurin treatment is the rapid (3-hr) decrease of c-Ki-*ras* RNA level (Fig. 7) that precedes commitment.

A similar trend was seen in c-*myc* mRNA expression to that found for c-*ras* transcripts, whereas expression of the amplified c-*abl* gene of K-562 cells (33) was not influenced (Z.K. and E.O., unpublished work). It has become increasingly common to find differentiated cells in which expression of c-*myc* protooncogene has decreased (34). This decrease might reflect an early response of this gene to cessation of cell proliferation, but alternatively down-regulation of c-*myc* gene might also be required for induction of the differentiation program. As discussed recently by Land *et al.* (35), the cooperation of *ras* and *myc* genes in displaying the malignant phenotype seems to be a significant juncture in the expression of neoplasia.

The results reported here suggest that *ras* gene products may serve important physiological function(s) in K-562 cells, functions that have a major role in mediating signal transduction by triggering the cascade of events that affect expression of the differentiation program.

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