IMP Dehydrogenase Inhibitor, Tiazofurin, Induces Apoptosis in K562 Human Erythroleukemia Cells

Marco Vitale,[1,2,*] **Loris Zamai,**[3] **Elisabetta Falcieri,**[2,4] **Giorgio Zauli,**[5] **Pietro Gobbi,**[3] **Spartaco Santi,**[2] **Caterina Cinti,**[2] **and George Weber**[6]

[1]Dipartimento di Scienze Biomediche e Biotecnologie, Sez. Anatomia Umana, Università di Brescia, Brescia, Italy

[2]Istituto di Citomorfologia Normale e Patologica CNR, c/o Ist. Codivilla Putti, Bologna, Italy

[3]Istituto di Anatomia Umana, Università di Bologna, Bologna, Italy

[4]Istituto di Anatomia e Fisiologia, Università di Urbino, Urbino, Italy

[5]Istituto di Anatomia Umana, Università di Ferrara, Ferrara, Italy

[6]Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis, Indiana

Tiazofurin, an anticancer drug which inhibits IMP dehydrogenase, decreases cellular GTP concentration, induces differentiation and down-regulates *ras* and *myc* oncogene expression, caused apoptosis of K562 cells in a time- and dose-dependent fashion. Apoptotic cells were detected by (1) flow cytometry, (2) electron microscopy, and (3) fluorescence in situ nick translation and confocal microscopy, while the DNA ladder was not detectable. The induced apoptosis was abrogated by guanosine which replenishes GTP pools through the guanosine salvage pathways, while it was enhanced by hypoxanthine, a competitive inhibitor of GPRT. The tiazofurin-mediated apoptosis may therefore be linked with the decrease of GTP and the consequent impairment of specific signal transduction pathways. Tiazofurin induced apoptosis also in lymphoblastic MOLT-4 cells, suggesting that this action is not confined to cells of the myeloid lineage, where the differentiating effects of the drug are more pronounced. Cytometry 30:61–66, 1997.   © 1997 Wiley-Liss, Inc.

Key terms: tiazofurin; apoptosis; K562; differentiation

INTRODUCTION

Several anticancer drugs have been reported to induce apoptosis in different cancer cells (1,2). Most of them perturb cell biochemistry, suggesting that different types of stimuli can generate common signals able to activate the apoptotic program. However, it is now accepted that apoptosis is a gene-directed process, representing one of the possible active responses a cell can give to external stimuli, and that at least some of the genes known to play a role in proliferation and differentiation, may be important in apoptosis as well (3–7). Tiazofurin (NSC 286193) is an anti-tumor nucleoside that is converted, in sensitive cells, to the active metabolite thiazole-4-carboxamide adenine dinucleotide (TAD), an NAD analogue, that potently inhibits IMP dehydrogenase (EC 1.1.1.205) leading to a depletion of GTP concentration. Tiazofurin has also been reported to reduce the proliferation rate of HL-60 and K562 cells in vitro and to induce erythroid differentiation in K562 cells and myeloid differentiation in leukemic patients (8,9). Moreover, tiazofurin was shown to decrease c-*myc* and c-*ras* expression in K562 cells and in the blast cells of patients with chronic granulocytic leukemia in blast crisis (8,10). All these phenomena are dependent on the reduction of cellular GTP concentration since they can be prevented by the addition of exogenous guanosin that,

through the salvage pathway, circumvents the IMP dehydrogenase inhibition by restoration of guanylate levels.

Here we report that tiazofurin induces apoptosis in K562 cells and like induced differentiation and reduction of oncogene expression, the tiazofurin-induced apoptosis is also prevented by the addition of guanosine. By contrast, hypoxanthine, a competitive inhibitor of GPRT, which salvages guanosine to GMP, enhances tiazofurin induced apoptosis in K562 cells. This suggests that apoptosis is linked to the perturbation of GTP metabolism.

MATERIALS AND METHODS
Cell Culture and Drug Administration

Human chronic myelogenous leukemia K562 cells (11) and human acute lymphoblastic leukemia MOLT-4 cells were routinely cultured in RPMI 1640 + 10% FCS, at 37°C, 5% CO_2. K562 cells/ml (2×10^5) were treated with 10 µM tiazofurin, 10 µM tiazofurin + 75 µM guanosine (Sigma Chemical Co., St. Louis, MO), or 10 µM tiazofurin + 100 µM hypoxanthine (Sigma Chemical Co.). Tiazofurin was prepared in stock aliquots (2 mM) of 150 µl in physiologi-

cal saline and kept at −20°C, until administration. Guanosine and hypoxanthine were dissolved in 0.1 N NaOH, adjusted to the concentration of 30 mM and used immediately. K562 cells were incubated in the presence of tiazofurin with or without guanosine or hypoxanthine up to 96 h, and cell cycle and erythroid differentiation were monitored every 24 h. MOLT-4 cells were treated with 10 μM tiazofurin, as described above.

Haemoglobin Determination

Erythroid differentiation of K562 cells was evaluated by haemoglobin synthesis as revealed by the benzidine reaction as previously described (8,12). Briefly, benzidine staining solution was freshly prepared by adding 5 μl of 30% hydrogen peroxide to 1 ml of stock solution of 2% benzidine, 0.5% acetic acid. The staining solution was diluted 1:1 with the cell suspension and, after 25 min, benzidine positive cells were counted under the microscope for a total of 300 cells. Differentiation was monitored daily up to 4 days in K562 cells untreated or treated with two doses of tiazofurin (5 and 10 μM) and tiazofurin (10 mM) + guanosine (75 μM) or hypoxanthine (100 μM).

Detection of Apoptosis

Apoptotic K562 cells were detected by several methods:

1. DNA gel electrophoresis: 5×10^7 K562 cells/ml were resuspended in lysis buffer containing: 0.1M EDTA, pH 8, 1M Tris-HCl, pH 7.8, 4M NaCl, 0.6% SDS, and added proteinase K to a final concentration of 100 μg/ml. DNA was extracted by the phenol/clorophorm method, as described (13) and analysed on a 0.8% agarose gel in $1 \times$ TBE buffer. Molecular weight marker III was from Boehringer Mannheim (Indianapolis, IN).

2. Flow cytometry: K562 cells were incubated 1.5 hr with 40 μg/ml propidium iodide. Apoptotic cells were detected as a PI^{dim} population as described (14,15) (Fig. 1).

3. Electron microscopy: for conventional TEM, control and treated K562 and MOLT-4 cells were sedimented at 24, 48, and 72 h and immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). They were then postfixed with 1% OsO_4 in veronal buffer, ethanol dehydrated and embedded in araldite as described (16). Formvarcarbon coated grids were used as thin section supports. Thin sections stained with uracyl acetate and lead citrate were observed with a Philips CM10 electron microscope at 80kV (Philips, Mahwah, NJ). A quantitative evaluation of apoptosis was obtained on toluidine blue semithin sections, on a total of 200 cells/sample.

4. Fluorescence in situ nick translation and confocal microscopy: the methodology described by De La Torre et al. (17) was followed. Briefly, after spreading of K562 cells fixed in methanol-acetic acid (3:1), the slides were incubated for 45 min at room temperature in a solution containing: 2 U of endonuclease-free DNA plymerase I and 10 μM dATP, dCTP, dGTP, and digoxigenin-11-dUTP in 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Slides were then washed for 5 min in 5% trichloroacetic acid at 4°C to remove unincorporated triphosphate nucleotides and rinsed in buffer I (1M Tris base, 1M NaCl,
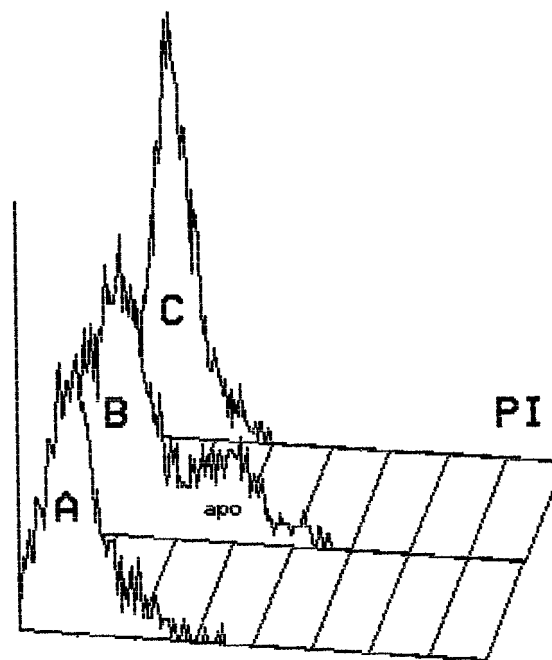

FIG. 1. Flow cytometry evaluation of apoptotic K562 by supravital exposure to PI. PI^{dim} fluorescence of K562 cells incubated 1.5 h with 40 μg/ml PI. Overlay of untreated (**A**), tiazofurin-treated (**B**), and tiazofurin + guanosine treated (**C**) samples after 72 h. Dimly fluorescent cells are apoptotic (apo). X axis: log scale.

20 mM $MgCl_2 \cdot 6H_2O$, pH 7.5). The reaction was blocked by incubation for 20 min at 42°C in buffer I containing 2% BSA, followed by 10 min in buffer I at room temperature. Samples were then labelled with anti-digoxigenin fluoresceinated antibody diluted 1:500 in buffer I with 2% BSA. The incubation was performed at room temperature for 45 min. Finally nuclei were counterstained with 1 μg/ml propidium iodide (PI) and observed by confocal microscopy. All biochemicals were obtained from Boehringer (Mannheim, Germany).

Observations were carried out with a Sarastro 1000 microscope (Molecular Dynamics, Sunnyvale, CA), equipped with an Argon ion laser attached to a Nikon Optiphot-2 camera. The detector was a photomultiplier and the pinhole in the front of the detector had a size of 50 μm. For the image acquisition, FITC and PI were excited simultaneously with the blue (488 nm) line of the argon laser, with the neutral density filters set at <30%. The emission signal was observed through a dichroic mirror (DM) (500 nm) followed by a 595 DM and a combination of a band-pass filter (530 ± 15 nm) and a cut-off filter (600 nm) to detect FITC and PI signals, respectively. The optical sections were obtained at increments of 0.3 μm in the Z-axis and stored on the computer (Silicon Graphic, Mountain View, CA; Personal IRIS-4D workstation) with a scanning mode format of 512×512 pixels. After Gaussian filtering, sections were vectorized and volume rendering was performed by VANIS (Volume Analysis Software System), to obtain the spatial projections, then superimposed and simultaneously analyzed.

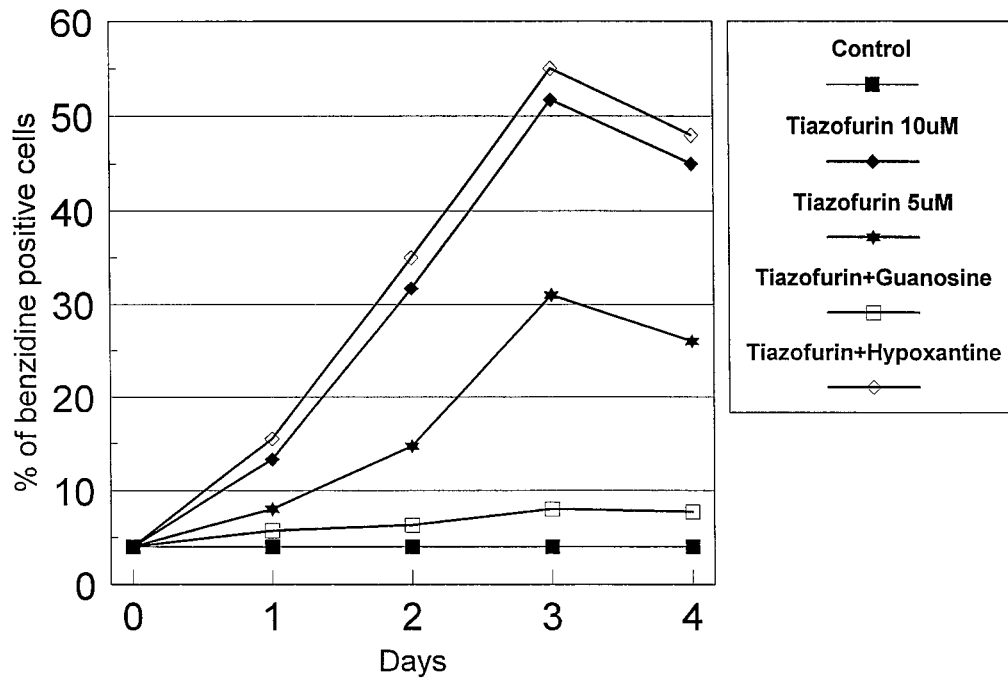## PERCENTAGE OF BENZIDINE POSITIVE K562 CELLS

FIG. 2. Kinetics of haemoglobin production in tiazofurin-treated K562 cells, as evaluated by benzidine reaction (initial value = 4%). Erythroid differentiation is abrogated by guanosine treatment. Means of three experiments are given (S.D. <7.6).
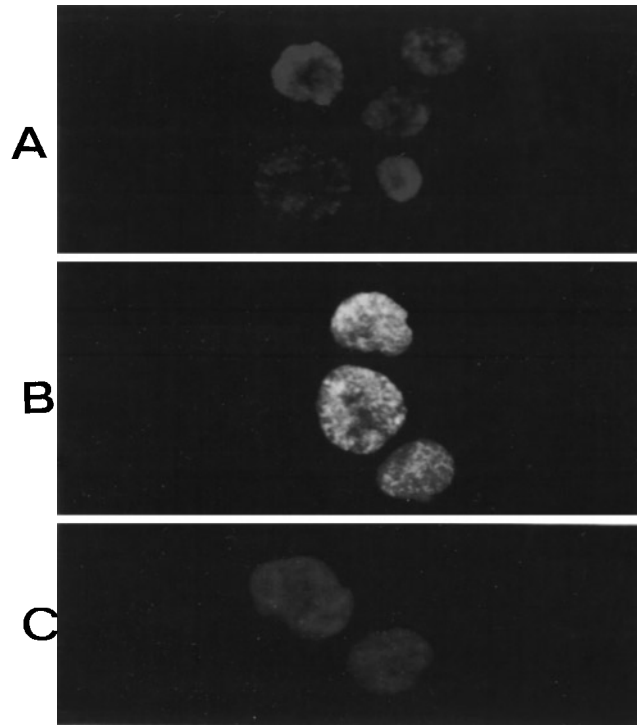

FIG. 3. Confocal microscopy of in situ nick translation in control (**A**), tiazofurin-treated (**B**), and tiazofurin + guanosine treated (**C**) K562 cells (×260). FITC signal (in green) of nicked nuclei and PI signal (in red). The nick translation areas are localized in whole nuclei (**B**). The superimposition of red and green signals appears yellow.
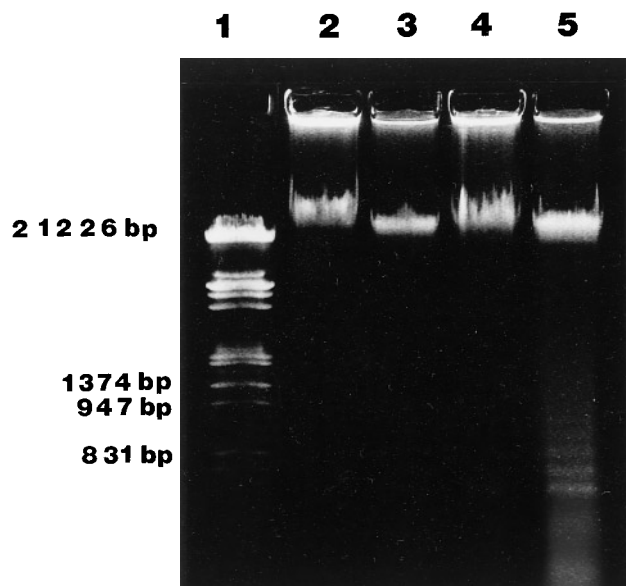
FIG. 4. DNA gel electrophoresis of untreated K562 cells (**lane 2**); 10 μM tiazofurin, 72 h (**lane 3**); 10 μM tiazofurin + 100 μM hypoxantine (**lane 4**). **Lane 5:** 0.15 μM camptothecin-treated HL-60 cells (6 h) (positive control for DNA fragmentation). **Lane 1:** Molecular weight markers λIII.

RESULTS

Tiazofurin retarded the K562 cell cycle progression, as expected (8). The reduction of S phase cell percentage induced by tiazofurin treatment was abrogated by guanosine (not shown). Along with S phase inhibition, tiazofurin induced a dose- and time-dependent erythroid differentiation that reached a peak at day 3 of treatment and was also prevented by guanosine (12), while hypoxanthine produced a small increase in the tiazofurin-induced erythroid differentiation (Fig. 2). Tiazofurin induced apoptosis in K562 cells (Fig. 1). We did not detect any spontaneous apoptosis in control K562 by any of the techniques we used. The nuclei of the untreated K562 cells appeared negative to the in situ nick translation (absence of yellow fluorescence in Fig. 3A). On the contrary, the presence of a yellow fluorescence in some nuclei of tiazofurin-treated K562 cells indicated a positive reaction to the in situ nick translation (Fig. 3B). Moreover, the nuclei of K562 cells treated with tiazofurin and guanosine appeared negative to the in situ nick translation (Fig. 3C). We were not able to detect DNA fragmentation by gel electrophoresis, even

using different extraction procedures. The "ladder" was not detectable in either tiazofurin or tiazofurin + hypoxanthine-treated samples (Fig. 4), independently from the concentration of the drug and the length of treatment. It is however well demonstrated (18) that the generation of a DNA ladder is not constant in apoptosis. The quantitative measurement of apoptosis as obtained by flow cytometry, is reported in Table 1. The detection of apoptotic cells as a PI^{dim} population (rather than the typical hypodiploid population) worked much better for cells (like K562) that are spontaneously hyperdiploid. The induction of apoptosis was dose- and time-dependent, and was prevented by guanosine addition. On the contrary, the addition of 100 μM hypoxanthine to 10 μM tiazofurin did enhance apoptosis in K562 cells. Hypoxanthine alone had no effect on K562 cells (not shown). In contrast to the regularly distributed heterochromatin observed in the control specimen (Fig. 5A), characteristically apoptotic nuclear changes appear in the tiazofurin treated samples. In fact, chromatin appears compacted in cup-shaped structures, sharply separated from the diffuse chromatin areas and marginated at the nuclear poles (Fig. 5B,C). Several nuclei appear, which undergo a strong condensing process, generating numerous electron dense micronuclei, scattered throughout the cytoplasm. Besides these alterations commonly present in all apoptotic cells, the ultrastructural analysis sometimes revealed an unusual nuclear behaviour in some K562 cells after 72 h treatment: a variable number of micronuclei, instead of being homogeneously electron-dense, showed a characteristic "moth-eaten" aspect, with electron transparent dots entirely surrounded by compact electron dense chromatin (Fig. 5D). Electron microscopy observation of tiazofurin (10 μM)-treated MOLT-4 cells for 72 h, showed the presence of a significant amount of apoptotic cells with respect to untreated controls (not shown).

DISCUSSION

Induction of apoptosis is an important cytotoxic mechanism of antineoplastic drugs. It requires the activation of a specific genetic program that is still poorly understood. Some genes whose products are known to play major roles in cell cycle regulation, cell differentiation, and signal transduction, are involved in the commitment of apoptosis (3–7), strongly suggesting that proliferation, differentiation, and programmed cell death are all part of the intricate mechanism of tissue growth and development. Tiazofurin through TAD, the active metabolite, acts as a specific IMP dehydrogenase inhibitor that depletes GTP pools. It has

Table 1
Percentage of Apoptotic Cells as Evaluated by Flow Cytometry: Kinetics of Apoptosis Induction in Tiazofurin-Treated K562 Cells and Synergistic Effect of Hypoxantine[a]

Hours	Control	Tiazofurin (10 μM)	Tiazofurin (5 μM)	10 μM Tiazofurin + 75 μM guanosine	10 μM Tiazofurin + 100 μM hypoxanthine
24	0	3.6 ± 2.2	0.9 ± 0.4	0.4 ± 0.2	4.6 ± 0.6
48	0	4.2 ± 2.1	1.9 ± 0.4	0.7 ± 0.2	13.0 ± 1.3
72	0	10.9 ± 3.4	2.9 ± 1.2	1.6 ± 1.2	15.4 ± 1.8
96	0	13.6 ± 3.2	4.2 ± 1.2	2.1 ± 1.5	20.2 ± 1.7

[a]Data are expressed as mean percentage ± S.D. of three separate experiments.
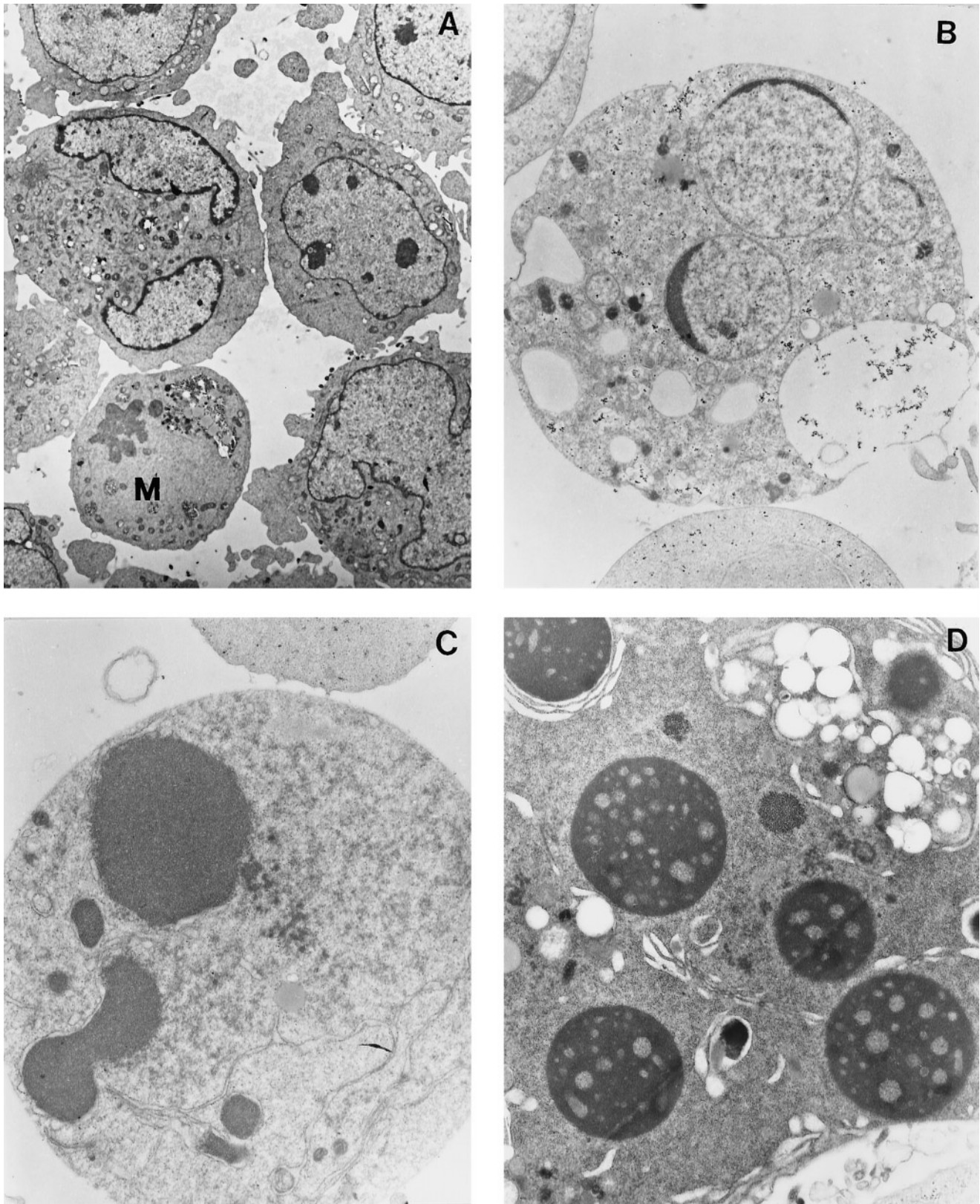
FIG. 5. Ultrastructural features of control (A) and tiazofurin-treated K562 cells for 24 h (B, C) and 72 h (D). Among the untreated normal cells (A), some mitoses (M) can be detected (×2,350). Cup-shaped chromatin marginations appear in the nuclei of an early apoptotic cell (B) (×4,600), while homogeneously electron dense micronuclei, still surrounded by well preserved cytoplasm and organelles, characterize the late stages (C) (×5,850). Micronuclei with "moth-eaten" morphology can be identified after longer treatments (D) (×5,400).

also been demonstrated to down-regulate *c-myc* and *c-ras* expression, to reduce Ras-GTP complexes, and to commit K562 cells to erythroid differentiation (10,19). Our results demonstrate that tiazofurin is also able to induce apoptosis in K562 cells at concentrations that are easily and routinely obtainable in vivo during the treatment of patients (10). Although cells of hematopoietic tumor cell lines when treated in vitro with anti-tumor drugs tend to die by apoptosis which, on the other hand, often represents the end-point of cell differentiation, we were not able to induce apoptosis in K562 cells by other agents, like campthotecin. In addition, the induction of apoptosis by tiazofurin had never been described before. DNA cleavage takes place within the first 24 h of treatment. Although we did not detect DNA breakage by gel electrophoresis, all apoptotic nuclei contain cleaved DNA as demonstrated by in situ nick translation. Tiazofurin-induced apoptosis of K562 cells can be abrogated by exogenous guanosine. On the contrary, the addition of hypoxanthine, that, inhibiting GPRT, prevents the salvage of guanosine to GMP, shows a synergistic effect with tiazofurin, suggesting a tight correlation between the commitment to apoptosis and the depletion of cellular GTP pools. Induction of apoptosis and erythroid differentiation do not seem to be necessarily related to each other nor mutually exclusive. Some apoptotic cells that are actively differentiating can, in fact, be detected after 72 h treatment, whereas at earlier treatment times the number of apoptotic and differentiating cells may be too low to reveal any significant correlation between the two phenomena. The observation that tiazofurin is also able to induce apoptosis in MOLT-4 cells suggests that this mechanism of action is not confined to cells of the myeloid lineage. It is well known that oncogenes can sensitize cells to apoptosis. In particular, activation of *c-myc* is a pre-requisite for the induction of apoptosis in many cell types (20–23); *ras* activation has been reported to make cells susceptible to apoptosis as well (24). Tiazofurin down-regulates *c-myc* and *c-ras* expression in K562 cells and retards their entrance S phase. When activated upon binding with GTP, *ras* products can mediate a number of cell functions, such as mitogenicity and early response genes (8,19–25) and take part in the complex mechanism of signal transduction. Ras-GTP complexes are decreased in K562 cells upon treatment with tiazofurin in a time- and dose-dependent manner. Moreover, when Friend erythroleukemia cells are treated for 96 h with tiazofurin, the induction of erythroid differentiation is accompanied by changes in the nuclear amount of phosphatidylinositol and phosphatidylinositol 4,5 bisphosphate due to the down-modulation of the β_1 isoform of PLC, present in the nucleus (26). Tiazofurin-mediated apoptosis could therefore be correlated with the impairment of specific signal transduction pathway activities that are reduced by this drug.

ACKNOWLEDGMENTS

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