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Induction of Apoptosis in Leukemic Cells by the Reversible Microtubule-disrupting Agent 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one: Protection by Bcl-2 and Bcl-X_L and Cell Cycle Arrest[1]

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ABSTRACT

We have found that the bicyclic colchicine analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC) induced a dose- and time-dependent apoptotic response in human leukemic cells. MTC and colchicine rapidly disrupted the microtubule integrity and arrested cells at the G_2-M phase before the onset of apoptosis. These responses were mediated by microtubule inhibition because 2-methoxy-5-[[3-(3,4,5-trimethoxyphenyl)propionyl]amino]-2,4,6-cycloheptatrien-1-one and lumicolchicine, inactive analogues of MTC and colchicine, respectively, were unable to promote microtubule disassembly, cell cycle arrest, and apoptosis. Although 1 μM MTC induced a complete microtubule disruption after 1 h of incubation in human leukemic HL-60 cells that led to an accumulation of cells at the G_2-M phase, MTC-induced apoptosis occurred after 9 h of treatment. This indicates the existence of a rather long lag between microtubule disruption and the onset of apoptosis. Unlike colchicine, the removal of MTC during this lag resulted in rapid microtubule repolymerization, followed by restoration of normal cell cycle and cell growth. MTC, but not 2-methoxy-5-[[3-(3,4,5-trimethoxyphenyl)-propionyl]amino]-2,4,6-cycloheptatrien-1-one, induced c-*jun* expression as well as c-Jun NH_2-terminal kinase and caspase activation, indicating that these signaling pathways are triggered by the specific action of MTC on microtubules. Caspase inhibition prevented MTC-induced apoptosis. Overexpression of *bcl*-2 or *bcl*-x_L by gene transfer in human erythroleukemic HEL cells abrogated MTC-induced apoptosis, but cells remained arrested in G_2-M, suggesting that *bcl*-2 and *bcl*-x_L block the signaling pathway between G_2-M arrest and triggering of apoptosis. MTC-treated *bcl*-2 and *bcl*-x_L-transfected HEL cells recovered their capacity to proliferate after MTC removal. These results indicate that microtubule inhibition induces G_2-M arrest and apoptosis in leukemic cells, showing a lag phase between G_2-M arrest and the onset of apoptosis, regulated by *bcl*-2 and *bcl*-x_L, during which MTC displays a reversible action on microtubule depolymerization and G_2-M cell cycle arrest. Thus, MTC is a potent apoptotic inducer on human leukemic cells and shows a remarkable reversible action on microtubule network and cell cycle before commitment for apoptosis is reached.

INTRODUCTION

Microtubules are cytoskeletal structures formed by highly dynamic assemblies of tubulin heterodimers, and they play a crucial role in many biological processes, including mitosis, intracellular transport, exocytosis, and cell growth. An essential function of microtubules is to partition duplicated chromosomes into two daughter cells during cell division. Microtubule dynamics are dramatically increased during mitosis, are very sensitive to interferences, and thereby constitute a moving target in cancer chemotherapy (1). Several agents affecting microtubule assembly/disassembly have been shown to induce apoptosis in a wide variety of cells, and some of them show a potent antitumor activity. Paclitaxel (Taxol) is a highly active, microtubule-stabilizing drug with significant clinical activity against a variety of solid tumors, especially ovarian and breast carcinoma, and acute leukemia (2, 3). Paclitaxel binds and stabilizes microtubules, thereby suppressing their dynamics. This results in G_2-M arrest, microtubule bundling, and cell death (2–6). In contrast, the *Vinca* alkaloids bind unassembled tubulin, thereby preventing microtubule assembly and suppressing microtubule dynamics as well, thus blocking progression through the cell cycle. The alkaloid colchicine (Fig. 1), extracted from *Colchicum autumnale*, binds to the tubulin molecule, thereby inhibiting its assembly into microtubules and microtubule dynamics (1). Tubulin-colchicine binding is slow, strongly temperature-dependent, and practically irreversible (7). Interaction of colchicine with tubulin is attributable to the simultaneous binding of its trimethoxyphenyl A and 2-methoxytropone C rings, whereas the middle connecting B ring is involved in the peculiar binding kinetics characteristic of the colchicine-tubulin interaction (7, 8). It has been shown that colchicine at micromolar doses can induce apoptosis in a number of cells (9–11). Although colchicine shows antitumor properties (11, 12), its therapeutic use is hampered by its high toxicity, a problem that has led to the synthesis of a variety of colchicine derivatives (13). Colchicine binds to β-tubulin, apparently close to the α-β dimerization interface (14, 15). An essential feature for powerful substoichiometric inhibition of microtubule assembly by synthetic analogues binding to the colchicine site is a properly positioned oxygen atom in ring C (16, 17), whereas ring A and its methoxy groups serve to increase the binding affinity (18). The bicyclic colchicine analogue MTC[3] (Fig. 1), which was synthesized by Fitzgerald (19), contains the two essential parts of the colchicine molecule that are required for binding to the tubulin site, *i.e.*, the trimethoxyphenyl A and the 2-methoxytropone C rings (7, 8), and lacks the middle ring B of colchicine (Fig. 1). MTC has been shown to bind rapidly and reversibly to the high affinity colchicine binding site of the tubulin molecule, thereby inhibiting microtubule assembly substoichiometrically (20–22). It has been also reported that MTC inhibits in a reversible way microtubule assembly (23, 24), cell growth in porcine kidney Pk15 cells (23), and exocytosis of cytoplasmic granules in activated human neutrophils (24). In the present study, we have analyzed the apoptotic effect of bicyclic colchicine MTC on cancer cells as well as the molecular mechanisms involved in its action. Appropriate control experiments were carried out with lumicolchicine and MTPC (Fig. 1), two microtubule-inactive analogues of colchicine and MTC, respectively. When colchicine is

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[3] The abbreviations used are: MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; MTPC, 2-methoxy-5-[[3-(3,4,5-trimethoxyphenyl)propionyl]amino]-2,4,6-cycloheptatrien-1-one; PARP, poly(ADP-ribose) polymerase; GST, glutathione *S*-transferase; JNK, c-Jun NH_2-terminal kinase; z-Asp-DBMC, z-Asp-2,6-dichlorobenzoyloxymethylketone.

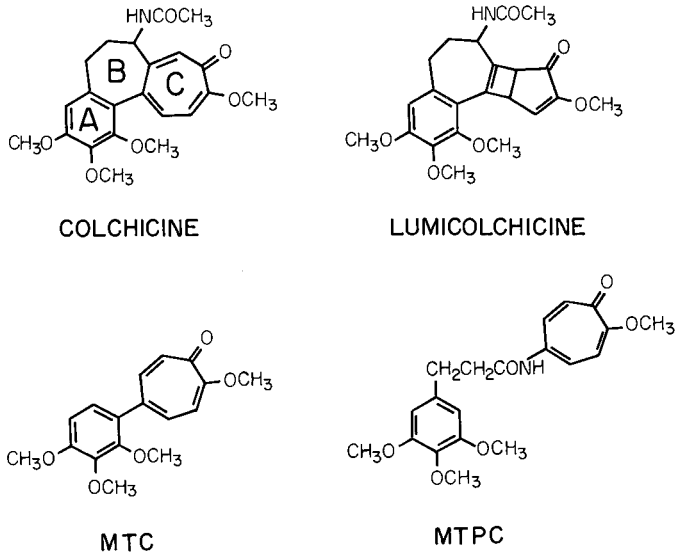

Fig. 1. Chemical structures of colchicine, lumicolchicine, MTC, and MTPC.

irradiated with long wavelength UV light, it is converted into lumicolchicine. Colchicine and lumicolchicine are structurally similar (Fig. 1), but lumicolchicine is inactive. The compound MTPC consists of the same trimethoxyphenyl and 2-methoxytropone moieties, but it is connected in a different manner by a propionamide spacer (Fig. 1). MTPC interacts weakly with tubulin and affects microtubule assembly only at high concentrations and therefore is considered as a conformationally inactive analogue of MTC (20, 23, 24).

We report here evidence indicating that MTC induces a rapid and reversible disruption of microtubules, leading to a G_2-M cell cycle arrest, that ultimately promotes apoptosis in human leukemic cells. We also show evidence for the existence of a reversible lag phase between microtubule disruption and the irreversible triggering of apoptosis in MTC-treated leukemic cells, during which both microtubule disruption and G_2-M cell cycle arrest can be reverted upon MTC removal. Furthermore, we show the sequence of events leading from microtubule inhibition to induction of apoptosis using a very specific reversible microtubule inhibitor.

MATERIALS AND METHODS

Chemicals and Reagents. MTC was a gift from Dr. T. J. Fitzgerald (Florida A & M University). MTPC was kindly provided by Dr. M. Gorbunoff (Brandeis University). Colchicine was purchased from Aldrich Chemical Co. (Steinheim, Germany). Paclitaxel (Taxol) was from Sigma Chemical Co. (St. Louis, MO). Lumicolchicine was prepared by long wavelength UV irradiation of colchicine. RPMI 1640 culture medium, FCS, antibiotics, and L-glutamine were purchased from Life Technologies, Inc. (Gaithersburg, MD). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham (Buckinghamshire, United Kingdom). DM1A anti-α tubulin monoclonal antibody was from Sigma. Mouse monoclonal antibody C2.10 against PARP was purchased from Enzyme Systems Products (Livermore, CA). Mouse monoclonal antibody Ab-1 against Bcl-2 was from Calbiochem (Cambridge, MA). Rabbit anti-Bcl-x_L antiserum was from Transduction Laboratories (Lexington, Kentucky). The caspase inhibitor z-Asp-DBMC was from Alexis (Läufelfingen, Switzerland). Guanidine thiocyanate was from Fluka (Buchs, Switzerland). Acrylamide, bisacrylamide, ammonium persulfate, and *N,N,N′N′*-tetramethylethylenediamine were from Bio-Rad (Richmond, CA). All other chemicals were from Merck (Darmstadt, Germany) or Sigma.

Cells and Culture Conditions. Human acute myeloid leukemia HL-60, human promonocytic leukemia U937, and human erythroleukemia HEL cells were grown in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 24 μg/ml gentamicin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. HEL cells were transfected by electroporation with the SFFV-Neo expression vector containing the human *bcl-2* or the human *bcl-x*$_L$ open reading frame driven by the long terminal repeat of the splenic focus-forming virus (pSFFV-*bcl-2* or pSFFV-*bcl-x*$_L$) as described previously (25) and selected by growth in the presence of 1 mg/ml G418. As a control, transfection was performed with empty SFFV-Neo plasmid. Microtubule-disrupting agents were added to the cell culture at the concentrations and for the times indicated in the respective figures. The caspase inhibitor z-Asp-DBMC (50 μM) was added 15 min before MTC treatment.

Immunofluorescence. HL-60 cells were plated onto 9 × 9-mm glass coverslips at a density of 3×10^5 cells/ml in the presence or absence of drugs for the desired concentration and time. Then, cells were centrifuged onto coverslips and processed as described previously (26). Cytoskeletons were fixed with 3.7% (w/v) formaldehyde-1% DMSO in PEM [100 mM piperazine-*N,N′*-bis(2-ethanosulfonic acid), 1 mM EGTA, and 1 mM $MgCl_2$ (pH 6.8)] for 30 min, and immunofluorescence was performed with DM1A monoclonal antibody reacting with α tubulin and fluoresceinated goat antimouse immunoglobulins as described previously (26). Cytoskeletons were observed with a Zeiss Axioplan epifluorescence microscope, and the images were recorded with a Hamamatsu 4724-95 cooled CCD camera.

Analysis of Apoptosis. To assess apoptosis, fragmented DNA was isolated, analyzed by electrophoresis on 1% (w/v) agarose gels, and stained with ethidium bromide as described previously (27). The induction of apoptosis was also monitored as the appearance of the sub-G_1 peak in cell cycle analysis (28). Briefly, cells (5×10^5) were centrifuged and fixed overnight in 70% ethanol at 4°C. Then, cells were washed three times with PBS, incubated for 1 h with 1 mg/ml RNase A and 20 μg/ml propidium iodide at room temperature, and analyzed for the distinct cell cycle phases with a Becton Dickinson (San Jose, CA) FACScan flow cytometer. Apoptosis was also analyzed *in situ* by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling technique using the Fluorescein Apoptosis Detection System kit (Promega, Madison, WI) according to the manufacturer's instructions, labeling the 3′-OH ends generated by DNA fragmentation through incorporation of fluorescein-12-dUTP. Fluorescent cells were visualized with a Zeiss LSM 310 laser scan confocal microscope.

Phosphatidylserine Exposure. Phosphatidylserine exposure at the external surface of the cell was measured by the binding of FITC-labeled annexin V according to the protocol outlined by the manufacturers in the Annexin-V-FLUOS reagent (Boehringer Mannheim, Mannheim, Germany). Then, cells were analyzed with a Becton Dickinson FACScan flow cytometer.

[^{3}H]Thymidine Incorporation. HL-60 cells (1.25×10^5 cells/ml) were incubated in 96-well plates with 200 μl of culture medium in the presence and in the absence of microtubule-disrupting agents and pulsed with [^{3}H]thymidine (0.1 μCi/well) for 24 h. Cells were then harvested in glass fiber filters using an automatic cell harvester. Filters were washed three times with distilled water, and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. All incubations were performed in triplicate.

Northern Blot. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNA (20 μg) was electrophoresed on 0.9% (w/v) agarose-formaldehyde gels and then transferred to Hybond-N nylon membranes (Amersham) as described previously (29). ^{32}P-labeled cDNA probes were prepared using the random hexanucleotide priming method (Oligo-Labeling kit; Pharmacia Biotech, Inc., Uppsala, Sweden) to a specific radioactivity $\geq 7 \times 10^8$ cpm/μg of cDNA. cDNA probe for c-*jun* (30) was kindly provided by Dr. R. Bravo (Squibb Institute, Princeton, NJ). The plasmid pAc 18.1, used as a probe for β-actin, was used as a control (30). Conditions for blot hybridization and washing have been described elsewhere (29). Quantitative analysis of the autoradiograms was performed by integration of peak areas after scanning with a PDI computing densitometer (Pharmacia).

Solid Phase JNK Assay. Protein kinase assays were carried out using a fusion protein between GST and c-Jun (amino acids 1–223) as a substrate of JNK, as described previously (31, 32) with slight modifications. Cells ($3–5 \times 10^6$) were resuspended in 200 μl of extract buffer [25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin]. Cells were incubated for 30 min in continuous rotation at 4°C and then microfuged at 12,000 rpm for 10 min.

Pellets were discarded and the supernatants, representing cell extracts, were diluted with 600 μl of dilution buffer [20 mM HEPES (pH 7.7), 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin]. Mixtures were incubated for 10 min on ice and then microfuged at 12,000 rpm for 10 min. The cell extracts were mixed with 20 μl of a suspension in dilution buffer of glutathione-agarose beads, to which GST-c-Jun were freshly bound. Mixtures were rotated overnight at 4°C in an Eppendorf tube and pelleted by centrifugation at 12,000 rpm for 1 min. After 4 × 1-ml washes in dilution buffer containing 50 mM NaCl, to remove kinases that have weaker affinity to bind c-Jun(1–223) than JNK, the pelleted beads were resuspended in 30 μl of kinase buffer [20 mM HEPES (pH 7.7), 2 mM DTT, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 20 μM ATP] and incubated with 4 μCi [γ-^{32}P]ATP. After 1 h at 30°C, the reaction was terminated by washing with dilution buffer containing 50 mM NaCl and microfugation at 12,000 rpm for 1 min. Then, the beads were boiled with 10 μl of 5× SDS-polyacrylamide gel sample buffer to elute the phosphorylated proteins, which were subsequently resolved in a SDS-10% polyacrylamide gel, followed by autoradiography. These conditions have been shown previously to enable specific binding of JNK to the c-Jun NH_2-terminal domain (31).

Western Blot Analysis. About 6 × 10^6 cells were pelleted by centrifugation, washed with PBS, lysed, and subjected to Western blot analysis as described previously (32). Briefly, proteins (20 μg) were run on SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose filters, blocked with 2% powder defatted milk, and incubated with mouse monoclonal anti-PARP antibody, mouse monoclonal anti-Bcl-2, or rabbit anti-Bcl-x_L antiserum. Then, signals were developed using an enhanced chemiluminescence detection system (Amersham).

RESULTS

Inhibition of Cell Proliferation and Induction of Apoptosis by MTC and Colchicine. We found that both MTC and colchicine (Fig. 1) inhibited cell growth of HL-60 cells in a dose-dependent manner (Fig. 2*A*). Incubation of cells in the presence of MTC or colchicine in the concentration range of 0.1–10 μM induced a significant inhibition of DNA synthesis as determined by [^{3}H]thymidine incorporation (Fig. 2*A*). HL-60 cells treated with 1 μM colchicine or MTC underwent apoptosis in a time-dependent manner, which was evident after 9 h of treatment (Fig. 2*B*). Control experiments were carried out with the microtubule-inactive analogues of colchicine and MTC, lumicolchicine and MTPC, respectively, showing no effect either on cell proliferation (Fig. 2*A*) or apoptosis (Fig. 2*B*). This indicated that the antimitogenic and apoptotic actions of both MTC and colchicine were attributable to their interaction with microtubules. The induction of apoptosis by MTC (Fig. 2*C*) or colchicine (data not shown) was dose-dependent, which was evident after treatment with 0.1 or 1 μM of the microtubule-disrupting agent. Similar results on induction of apoptosis by MTC or colchicine were obtained using the human promonocytic leukemic U937 cells (data not shown). The apoptotic response induced by MTC on HL-60 cells was also evidenced by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (data not shown) and by phosphatidylserine exposure on the outer leaflet of the cell surface measured through FITC-annexin V binding (data not shown).

Reversible Microtubule Disruption in MTC-treated Cells. One μM MTC and colchicine induced a rapid depolymerization of cytoplasmic microtubules in HL-60 cells (Fig. 3). The effect is noticeable with as short as a 15-min incubation, and it reaches practically complete microtubule depolymerization after 1 h of incubation with each drug (Fig. 3). However, the inactive analogues MTPC and lumicolchicine were unable to promote disassembly of microtubules (data not shown). Microtubule repolymerization was analyzed in colchicine- and MTC-treated HL-60 cells after drug removal. HL-60 cells treated with 1 μM MTC or colchicine for 6 h showed a complete

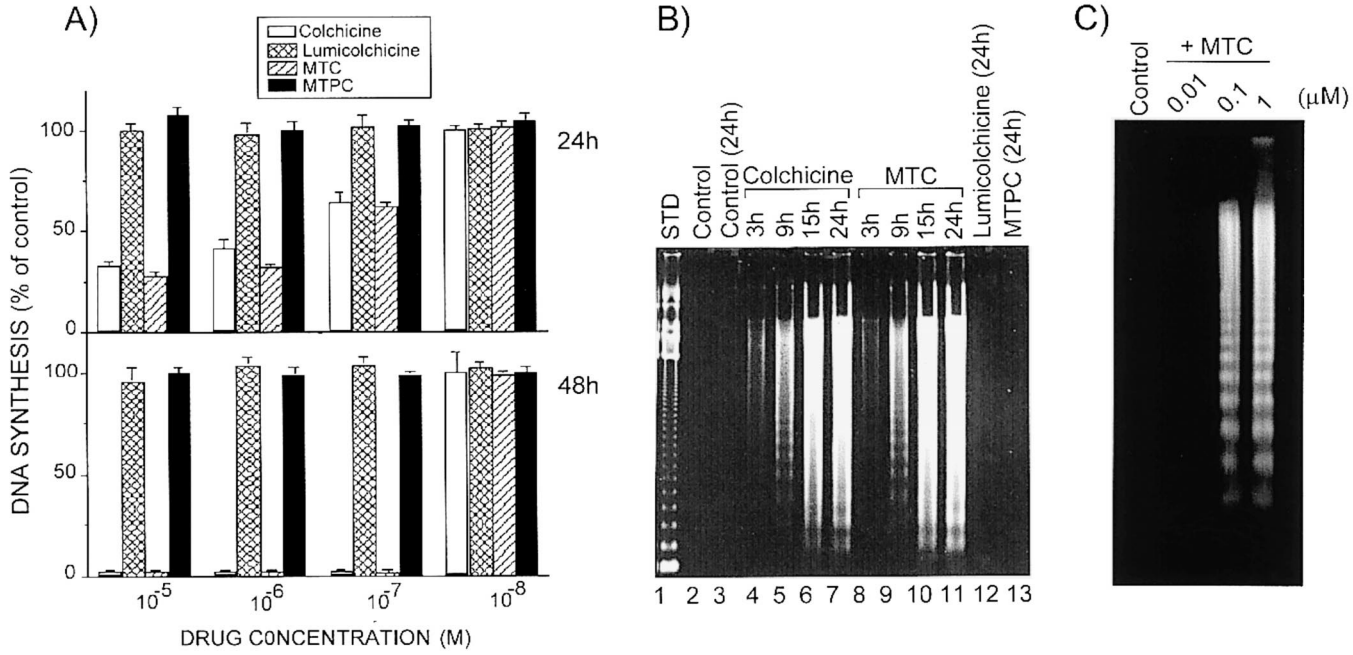

Fig. 2. Effect of colchicine, lumicolchicine, MTC, and MTPC on the cell growth and induction of apoptosis in HL-60 cells. *A*, growth inhibition in colchicine- and MTC-treated HL-60 cells. Cells were incubated with increasing concentrations of colchicine, lumicolchicine, MTC, and MTPC for the times indicated, and [^{3}H]thymidine incorporation into DNA was measured as described in "Materials and Methods." Results are expressed as the percentage of the cpm incorporated in untreated HL-60 cells. Data are shown as means of three independent experiments ± SD. *B*, time course of colchicine- and MTC-induced apoptosis in HL-60 cells. Cells were treated with 1 μM of the compounds indicated, and the fragmented DNA was extracted and analyzed at different times as described in "Materials and Methods." *Lane 1*, 123-bp DNA ladder used as standard (*STD*); *Lanes 2* and *3*, untreated control cells collected at time zero and after 24 h of cell culture; *Lanes 4–7*, cells treated with colchicine for 3, 9, 15, and 24 h; *Lanes 8–11*, cells treated with MTC for 3, 9, 15, and 24 h; *Lane 12*, cells treated with lumicolchicine for 24 h; *Lane 13*, cells treated with MTPC for 24 h. Results shown are representative of three independent experiments performed. *C*, dose response of MTC-induced DNA fragmentation. HL-60 cells were incubated for 15 h with increasing concentrations of MTC and assayed for DNA fragmentation as described in "Materials and Methods." Untreated control cells (*Control*) were run in parallel in the same gel. Results shown are representative of three independent experiments performed. Fragmented DNA from 6 × 10^5 cells was loaded in each lane of the agarose gels shown in *B* and *C*.

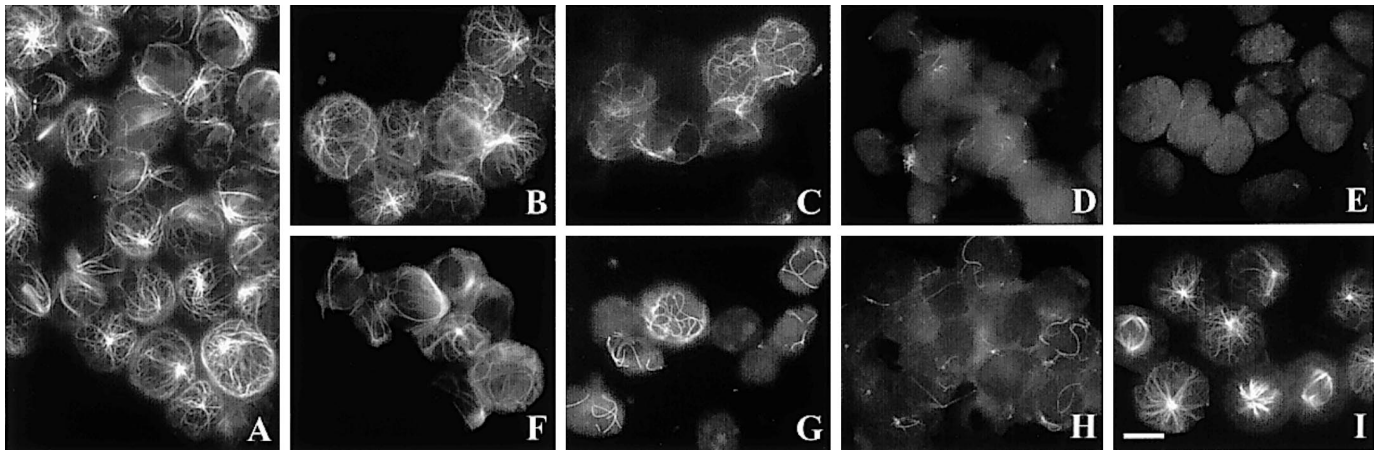

Fig. 3. Time course of the effect of colchicine and MTC on the microtubule network of HL-60 cells. Cells were incubated in the absence (*A*) or in the presence (*B–E*) of 1 μM colchicine or 1 μM MTC (*F–I*) for 15 min (*B* and *F*), 30 min (*C* and *G*), and 1 h (*D* and *H*) and then fixed and processed for immunofluorescence of microtubules as described in "Materials and Methods." Cells incubated for 6 h with 1 μM colchicine or 1 μM MTC, washed, and then incubated in drug-free culture medium for 30 min showed an almost complete restoration of the microtubule network and appearance of mitosis in the case of MTC-treated cells (*I*), whereas the colchicine effect was not reversed (*E*). *Bar*, 10 μm. The photomicrographs shown are representative of at least three independent experiments performed.

absence of cytoplasmic microtubules, leading to a microtubule fluorescence image identical to that observed after MTC or colchicine treatment for 1 h (Fig. 3, *D* and *H*). Then, MTC- and colchicine-treated HL-60 cells for 6 h were washed three times and placed in fresh culture medium in the absence of drugs to analyze whether the effect on microtubule depolymerization could be reverted. Regeneration of the microtubule network was observed shortly after removal of MTC from the cell culture medium, which was almost complete after 30 min (Fig. 3*I*). In contrast, the action of colchicine was not reverted (Fig. 3*E*), and microtubule repolymerization was not observed even after 6 h incubation in colchicine-free culture medium.

Mitotic Arrest in MTC-treated Cells Occurs before the Onset of Apoptosis and Can Be Reversed Upon MTC Removal. To assess the extent of MTC-induced apoptosis and to have a quantitative view of the effect of MTC on the cell cycle, we analyzed the cell cycle distribution of HL-60 cells treated with 1 μM MTC using flow cytometry (Fig. 4*A*). After 30–60 min of incubation, MTC-treated cells started to accumulate in G_2-M, reaching a maximum after 6–9 h of incubation, with a parallel decrease in cells in G_0/G_1 (Fig. 4*A*). The appearance of cells with a DNA content less than G_1, characteristic of early apoptotic cells (sub-G_1 peak; Fig. 4*A*), could be observed after 9 h of treatment with 1 μM MTC together with a significant decrease in the proportion of cells in G_2-M (Fig. 4*A*). At 24 h of incubation with 1 μM MTC, about 66% of the cells underwent apoptosis (Fig. 4*A*). Similar results were obtained with colchicine (data not shown). As removal of MTC led to rapid reversion of the microtubule network (Fig. 3), we analyzed whether the changes observed in the cell cycle induced by MTC, before triggering of apoptosis, could be reversed upon its removal. HL-60 cells exposed to 1 μM MTC for 6 h showed an increase in G_2-M without a significant induction of apoptosis (Fig. 4, *A* and *B*). Then, cells were washed three times with culture medium to remove the drug and incubated in the absence of MTC until completion of the 24-h incubation time. Under these conditions, we observed an almost total recovery of the initial percentages of the distinct cell cycle phases, whereas cells treated with MTC for 24 h underwent a strong apoptotic response (Fig. 4*B*). A partial recovery of the normal cell cycle profile was detected as soon as 1 h after MTC removal, and a normal cell cycle distribution was detected after 9 h of MTC removal (data not shown). In contrast, colchicine removal did not allow cell cycle reversion, and cells were directed to apoptosis (data not shown). Thus, we found a reversible action of MTC on microtubule disruption and on cell cycle arrest, whereas colchicine behaved as an irreversible agent. In addition, we found that removal of MTC from HL-60 cells, previously treated with 1 μM MTC for 1, 3, or 6 h and then grown in MTC-free culture medium until completion of the 24 h incubation time, practically restored the normal HL-60 cell proliferative capacity (Fig. 4*C*). However, a significant inhibition in cell proliferation was observed after MTC removal from cells incubated for 9 h with MTC, when the apoptotic response has been already initiated, and then incubated in MTC-free culture medium (Fig. 4*C*). This indicates that the cell cycle arrest in G_2-M induced by MTC could be reverted by MTC removal providing that apoptosis is not triggered.

Induction of c-*jun* and c-Jun Kinase by MTC. Because c-*jun* has been implicated in the induction of apoptosis in various systems (32, 33), we analyzed the effect of MTC and colchicine on the expression of c-*jun* proto-oncogene in the human leukemic HL-60 cell line. The addition of 1 μM MTC to promyelocytic HL-60 cells induced a potent and sustained increase in the 2.7-kb c-*jun* steady-state mRNA level (Fig. 5*A*). Colchicine also induced a significant increase in c-*jun* expression (Fig. 5*A*). The increase in the level of c-*jun* transcripts induced by MTC and colchicine was maintained even after 8 h of treatment with both agents (data not shown). After 2 h of treatment, MTC and colchicine promoted a 7-fold and a 3-fold induction in the c-*jun* mRNA steady state level, respectively, using β-actin expression as an internal control. These actions of MTC and colchicine on gene expression were specific to their respective actions on microtubules because the microtubule-inactive analogues MTPC and lumicolchicine, respectively, showed no effect on c-*jun* expression (data not shown). Because c-*jun* proto-oncogene is positively autoregulated by its own gene product, once it is properly phosphorylated and JNK activates c-Jun transcriptional activity (31), agents that cause sustained induction of c-Jun are potential inducers of the JNK pathway. On the other hand, JNK activation has been suggested to be involved in the induction of apoptosis by a wide variety of agents and during development (32, 34–37). Because we have found that MTC induces apoptosis (Figs. 2 and 4) and expression of c-*jun* (Fig. 5*A*) in HL-60 cells, we next examined whether MTC was able to activate JNK in these cells. To determine JNK activation, we used a GST fusion protein containing amino acids 1–223 of c-Jun, GST-c-Jun-(1–223). This fusion protein was bound through its GST moiety to glutathione-agarose beads to generate an affinity matrix to precipitate JNK activities from HL-60 cell lysates. The precipitated complexes were washed and subjected to solid-phase kinase assay. As shown in Fig.

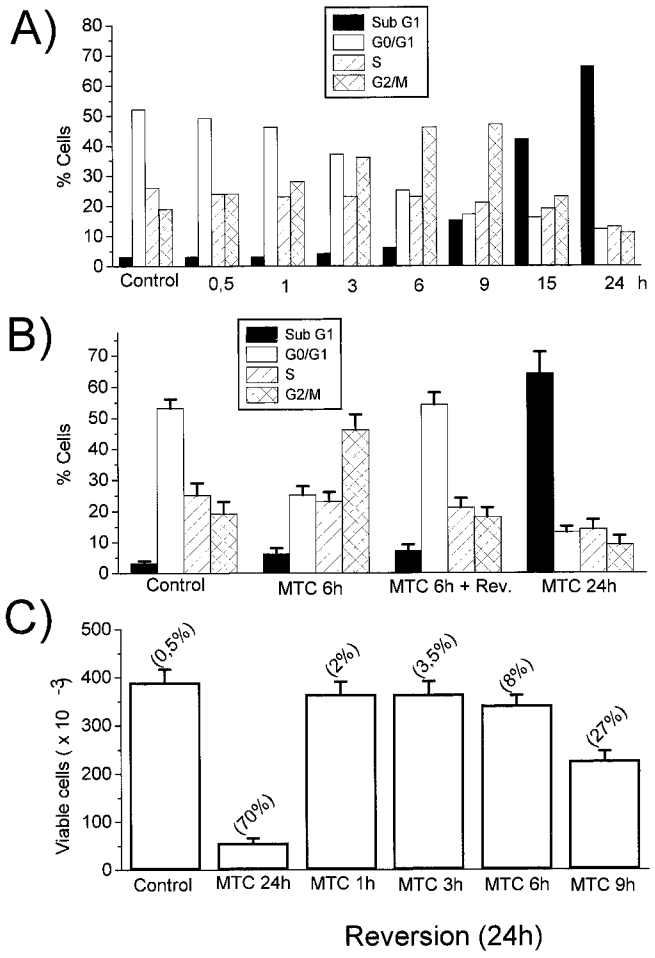

Fig. 4. Reversion of the effects of MTC on cell cycle distribution and growth of HL-60 cells. *A*, time course of MTC-induced effect on cell cycle. HL-60 cells were treated with 1 μM MTC. At the indicated time points, the proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Results shown are representative of four independent experiments performed. *B*, reversible effect of MTC on cell cycle arrest. Untreated HL-60 cells (*Control*), HL-60 cells treated with 1 μM MTC for 6 and 24 h, and HL-60 cells treated with 1 μM MTC for 6 h and then washed to remove the drug and grown in MTC-free culture medium until completion of the 24-h incubation time (*MTC 6 h + Rev.*) were analyzed for cell cycle by fluorescence flow cytometry, and the proportion of cells in each phase of the cell cycle was quantitated. Data are shown as means of four independent experiments ± SD. *C*, reversible effect of MTC on cell proliferation. HL-60 cells (1.8×10^5) were grown for 24 h in the absence of any drug (*Control*), treated with 1 μM MTC for 24 h, or treated with 1 μM MTC for 1, 3, 6, and 9 h and then grown in fresh MTC-free culture medium until completion of the 24-h incubation time. Then, viable cells, determined by trypan blue dye exclusion, were counted. The percentage of nonviable cells is shown in parenthesis. Data are shown as means of four independent experiments ± SD.

5*B*, GST-c-Jun phosphorylation was observed after 15–30 min of incubation with 1 μM MTC, when microtubule disruption is taking place, and the response was further increased with the incubation time and found to be persistent. A strong JNK activation was obtained after 1–3 h of treatment with MTC (Fig. 5*B*), long before DNA fragmentation, occurring after 9 h of treatment (Figs. 2 and 4). This high MTC-induced JNK activation was detected even after 8 h of treatment (data not shown). The inactive analogue MTPC was unable to induce JNK activation (data not shown), indicating that the MTC-induced JNK activation was attributable to the action of MTC on microtubules. In contrast to this persistent MTC-induced JNK activation, we have found a transient JNK activation, peaking at 15 min, when HL-60 cells were induced to differentiate toward the monocytic/macrophage lineage by tumor necrosis α (32). This further supports the notion that duration of JNK induction is regulated differentially in HL-60 differentiation and apoptosis (32).

Involvement of Caspase Activation in MTC-induced Apoptosis. An increasing number of cysteine proteases named caspases are required for the accurate and limited proteolytic events that typify programmed cell death. We found that MTC induces caspase activation as shown by the cleavage of the typical caspase-3 substrate PARP, using the anti-PARP C2.10 monoclonal antibody that detected both the 116-kDa intact form and the 85-kDa cleaved form of PARP (Fig. 6). Incubation with the caspase inhibitor z-Asp-DBMC blocked completely PARP degradation and the apoptotic response analyzed by DNA fragmentation in agarose gels and by flow cytometry (Fig. 6). The inactive analogue MTPC was unable to promote caspase activation (data not shown), indicating that the MTC-induced caspase activation was attributable to the action of MTC on microtubules.

Overexpression of *bcl-2* and *bcl-x_L* Inhibits MTC-induced Apoptosis. Because previous reports have shown that overexpression of Bcl-2 or Bcl-x_L inhibited paclitaxel-induced apoptosis (38, 39), we studied the role of Bcl-2 and Bcl-x_L in the induction of apoptosis by MTC in human erythroleukemia HEL cells. These cells were stably transfected with the expression vectors pSFFV-*bcl-2* or pSFFV-*bcl-x_L*, containing the human *bcl-2* or *bcl-x_L* open reading frame, respectively, or with control pSFFV-Neo plasmid. Western blot analysis indicated that HEL-Neo cells expressed endogenous Bcl-x_L and no Bcl-2, and the *bcl-2*- and *bcl-x_L*-transfected HEL cells overexpressed these two gene products (Fig. 7*A*) as previously reported (25). Fig. 7 shows that HEL-Neo cells underwent apoptosis upon treatment with MTC. However, overexpression of Bcl-2 by gene transfer in HEL cells led to a complete inhibition of MTC-induced apoptosis, even after 48 h of MTC treatment (Fig. 7). Similar results were obtained upon transfection with Bcl-x_L (Fig. 7). Nevertheless, overexpression

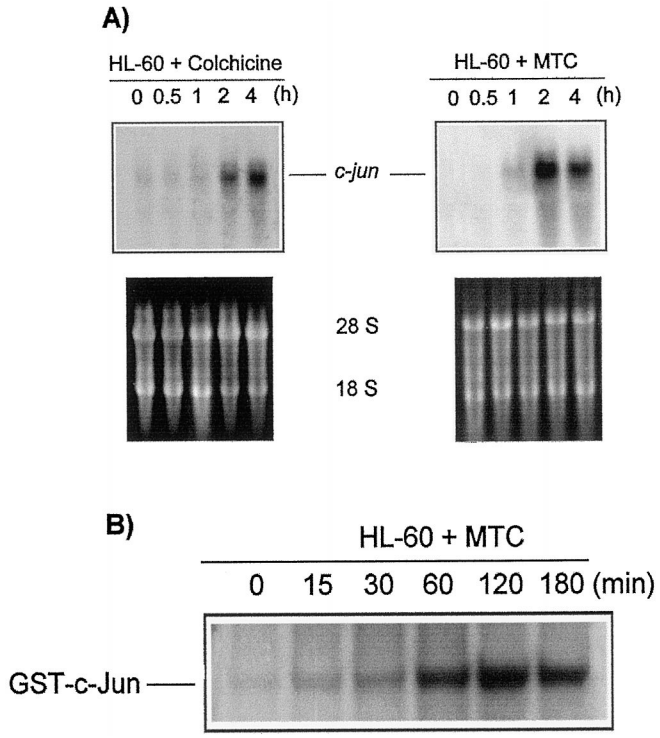

Fig. 5. Induction of c-*jun* expression and JNK activation by MTC in HL-60 cells. *A*, Northern blot analysis of mRNA levels after cell treatment with 1 μM colchicine or 1 μM MTC for the times indicated. Basal control levels in untreated HL-60 cells are also shown. rRNA staining with ethidium bromide (*bottom*) was used as the loading control. *B*, time course of MTC-induced JNK activation in HL-60 cells. Cells were treated with 1 μM MTC for the times indicated and assayed for JNK activation as described in "Materials and Methods." Control untreated cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Experiments shown are representative of three performed.

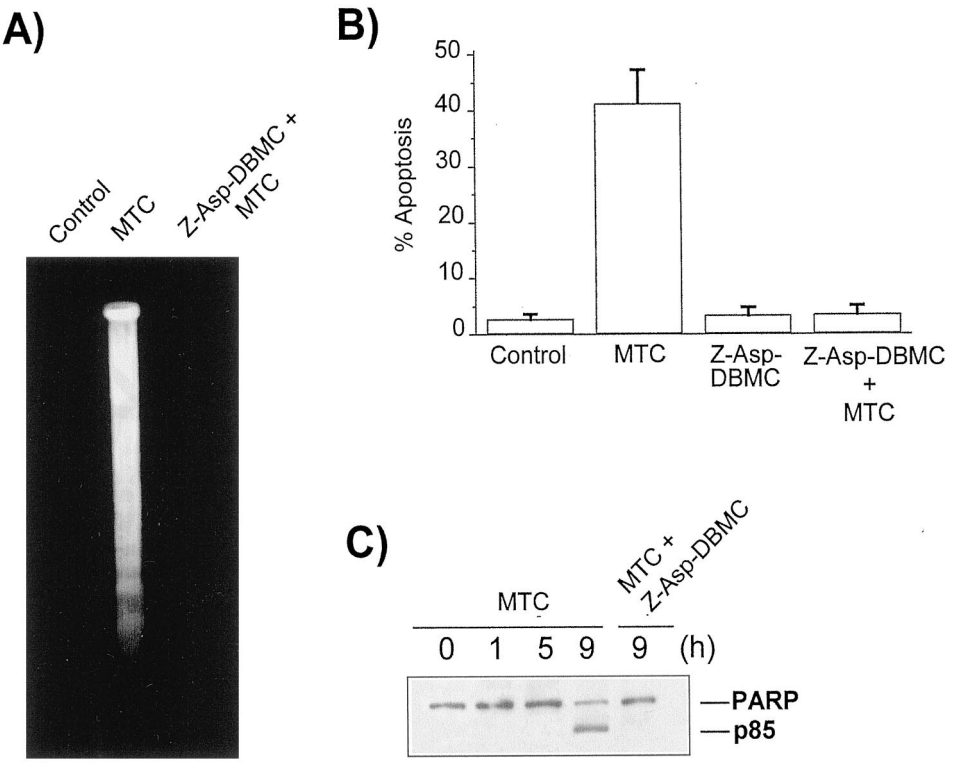

Fig. 6. Caspase inhibition prevents apoptosis and PARP degradation in MTC-treated HL-60 cells. HL-60 cells were incubated with 1 μM MTC in the absence or in the presence of 50 μM z-Asp-DBMC for 15 h (*A* and *B*) and then were analyzed for DNA fragmentation in agarose gels (*A*) or by flow cytometry (*B*) as described in "Materials and Methods." Fragmented DNA loaded in each lane of *A* was from 6×10^5 cells. Experiment shown in *A* is representative of three performed. Data shown in *B* are means of three independent determinations ± SD. Control untreated cells (*Control*) and cells treated only with the caspase inhibitor were also run in parallel. *C*, time course of MTC-induced PARP cleavage upon MTC treatment in HL-60 cells. Cells were treated with 1 μM MTC for the indicated times and subsequently lysed, subjected to SDS-8% PAGE, and immunoblotted with an anti-PARP monoclonal antibody as described in "Materials ands Methods." The migration position of full-length PARP and the cleavage product p85 are indicated. The experiment shown is representative of three performed.

of Bcl-2 (Fig. 7*C*) and Bcl-x_L (data not shown) did not affect the cell cycle arrest in G_2-M phase induced by MTC. As shown in Fig. 7*C*, *bcl-2*-transfected HEL cells accumulated in the G_2-M phase upon MTC treatment, and after 24 h of treatment, practically the whole cell population was arrested in the G_2-M phase. Bcl-2-transfected HEL cells remained arrested in G_2-M for prolonged periods of time (at least 48 h) without undergoing apoptosis (Fig. 7*B*). Thus, overexpression of Bcl-2 completely prevented MTC-induced apoptosis, but did not affect the cell cycle arrest in G_2-M promoted by MTC. Similar results concerning prevention of apoptosis and arrest in G_2-M were obtained in *bcl-2*-transfected HEL cells treated with colchicine or Taxol (data not shown). Interestingly, removal of MTC from Bcl-2- and Bcl-x_L-transfected HEL cell cultures that had been treated with MTC for 48 h led to reversion of the MTC effect on growth arrest and restored their growth capacity (Fig. 7*D*). Cell growth capacity was slowly recovered and initiated after about 24 h after MTC removal.

DISCUSSION

We have characterized in the present study the effect of the microtubule-disrupting compound MTC, a bicyclic colchicine analogue, on microtubule network, cell cycle, and apoptosis in human leukemic cells. Our data indicate that: (*a*) MTC induces apoptosis in human leukemic cells; (*b*) MTC induces a rapid and potent disruption of microtubules, G_2-M phase cell cycle arrest, and inhibition of cell proliferation before the onset of apoptosis; (*c*) MTC induces an increase in the steady-state mRNA level of c-*jun*; (*d*) MTC induces JNK and caspase activation; (*e*) inhibition of caspase activation prevents MTC-induced apoptosis; (*f*) overexpression of *bcl-2* or *bcl-*x_L abrogates MTC-induced apoptosis without affecting its cell cycle effects; and (*g*) unlike colchicine, removal of MTC from the culture medium before the onset of apoptosis resulted in microtubule repolymerization and in restoration of normal cell cycle distribution and cell growth. All these MTC actions were specific for its interaction with microtubules because the structurally microtubule-inactive analogue MTPC was unable to raise these responses. The reversible effects of MTC on microtubule network, cell cycle, and proliferation could be of potential importance regarding the synthesis of colchicine analogues with a lower toxicity in *in vivo* assays.

The data reported here establish the sequence of events leading from microtubule disruption to induction of apoptosis, using a very specific reversible microtubule inhibitor. We have found that there is a rather prolonged lag time between complete disruption of microtubules and triggering of apoptosis, during which microtubules can be reassembled, and normal cell cycle distribution and cell proliferation can be restored in MTC-treated HL-60 cells shortly after removal of MTC. We found that HL-60 cells treated with 1 μM MTC for 1–6 h were deprived completely of cytoplasmic microtubules, leading to a drastic change in cell morphology with plenty of surface protrusions (data not shown). However, no significant internucleosomal DNA breakdown was observed, and after MTC removal, cells rapidly repolymerized the microtubule network, recovered the original cell shape and cell cycle distribution, and grew normally. Thus, the results reported here indicate that MTC promotes a number of reversible actions on cells before commitment for apoptosis is triggered. The time spanning the initial MTC-induced effects and the irreversible apoptotic commitment depends on the drug concentration used (data not shown). Fig. 8 depicts the timing of the distinct effects elicited by 1 μM MTC on human leukemic HL-60 cells as well as the reversibility of the processes, based on the present results. The effect of MTC can be dissected in three critical steps. First, MTC induces a rapid microtubule depolymerization that culminates at 1 h of treatment. Second, MTC increases cell cycle arrest in G_2-M, reaching a significant G_2-M arrest by 6 h of incubation. Third, MTC promotes ultimately the internucleosomal DNA degradation, a hallmark of apoptosis, after 9 h incubation. The first two steps are reversible upon MTC removal, whereas the third one involves the irreversible phase of MTC action leading to apoptosis. Overexpression of Bcl-2 and Bcl-x_L efficiently inhibited apoptosis induced by MTC as well as by other microtubule-

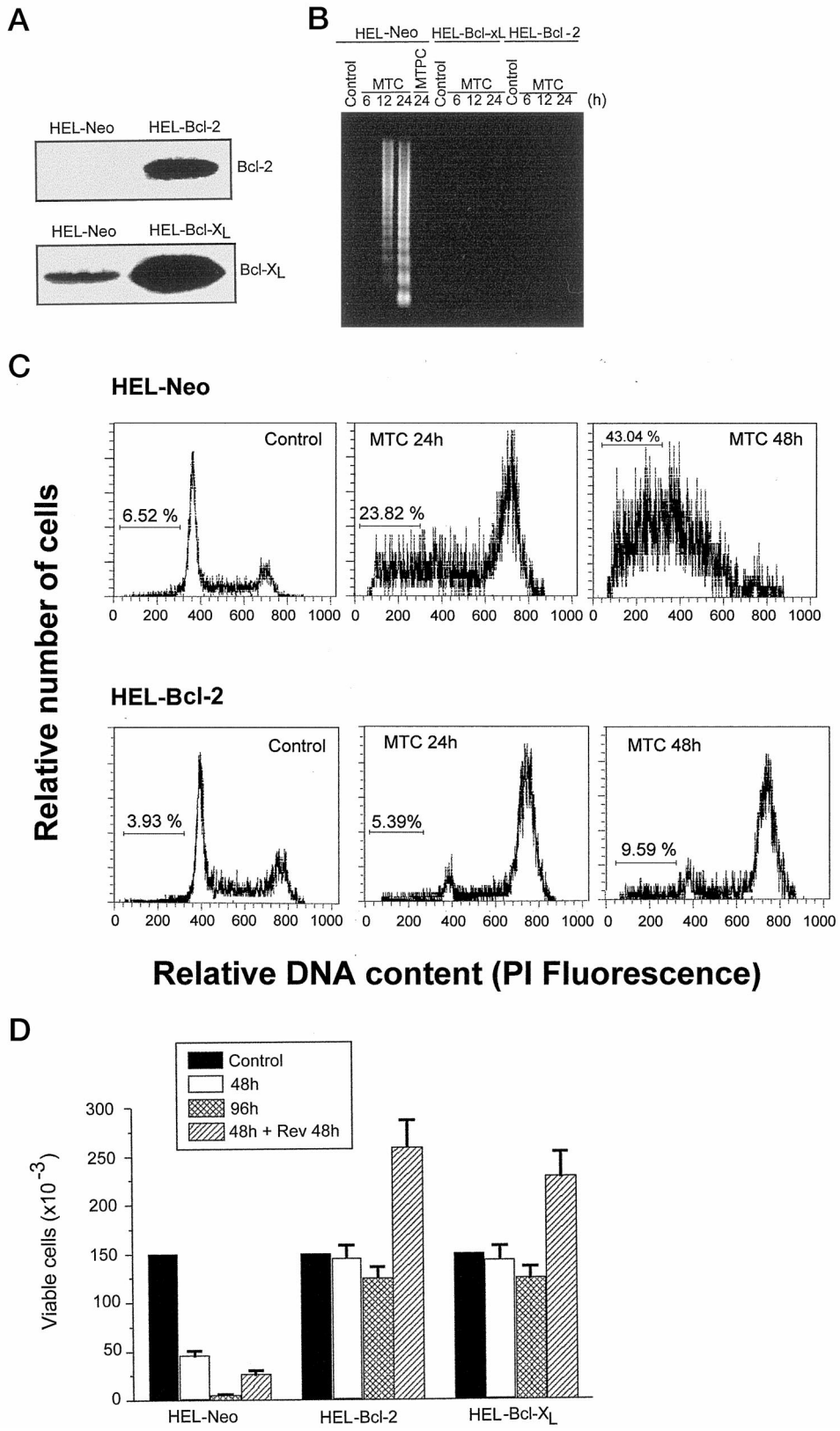

Fig. 7. Prevention of MTC-induced apoptosis by overexpression of Bcl-2 or Bcl-x_L. *A*, Western blot analysis for Bcl-2 and Bcl-x_L. Cell lysates from Neo, Bcl-2-transfected, and Bcl-x_L-transfected HEL cells were loaded onto a SDS-14% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose filters and analyzed for Bcl-2 and Bcl-x_L as described in "Materials and Methods." *B*, analysis of DNA fragmentation in HEL-Neo, HEL-Bcl-2, and HEL-Bcl-x_L cells after treatment with 1 μM MTC for the times indicated. Fragmented DNA was extracted and analyzed as described in "Materials and Methods." Fragmented DNA from 6×10^5 cells was loaded in each lane. The experiment shown is representative of three performed. Untreated control cells (*Control*) and cells treated with 1 μM MTPC were run in parallel. *C*, effect of MTC on the cell cycle distribution of HEL-Neo and HEL-Bcl-2. Representative examples of the cell cycle effects of 1 μM MTC on HEL-Neo and HEL-Bcl-2 cells are shown. Untreated cells (*Control*) or cells treated with MTC were stained with propidium iodide, and their DNA content was analyzed by fluorescence flow cytometry. The percentage of cells with a DNA content less than G_1 (sub-G_1) is indicated in each histogram. Data shown are representative of three experiments performed. *D*, reversible effect of MTC on cell growth. HEL-Neo, HEL-Bcl-2, and HEL-Bcl-x_L were cultured at 1.5×10^5 cells/ml (*Control*) and treated with 1 μM MTC for 48 h or 96 h, or treated with 1 μM MTC for 48 h, and then washed and incubated in fresh MTC-free culture medium for an additional 48 h. Viable cells, determined by trypan blue dye exclusion, were counted. Data are shown as means of three independent experiments ± SD.

active agents, such as colchicine and Taxol, without affecting their actions on the cell cycle, *i.e.*, G_2-M arrest. This indicates that G_2-M arrest is before the induction of apoptosis in MTC-treated cells and suggests that cells can be arrested at this point without going into the apoptotic phase if enough expression of antiapoptotic genes is present. These MTC-treated Bcl-2- and Bcl-x_L-transfected cells can continue to proliferate after MTC removal. Putative mechanisms by which Bcl-2 and Bcl-x_L interfere with signaling between G_2-M arrest and apoptosis onset include prevention of mitochondrial transmembrane potential collapse, cytochrome *c* release, and caspase-9 activation

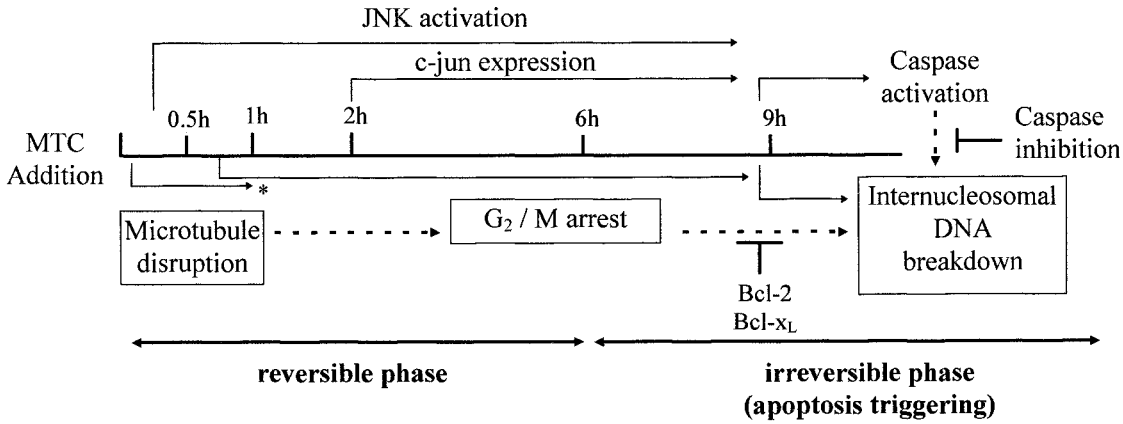

Fig. 8. Timing of the MTC effects on HL-60 cells. This is a schematic diagram designed to portray the sequence of events that we have detected in HL-60 cells treated with 1 μM MTC. Microtubule depolymerization constitutes the first specific action of MTC. *, indicates that disruption of microtubule disruption is practically complete after 1 h treatment. The biochemical events triggered by MTC in a chronological order are: JNK activation, c-*jun* induction, and caspase activation. The physiological processes elicited by MTC (in chronological order: microtubule disruption, G_2-M arrest, and induction of apoptosis) are placed in *boxes*. Overexpression of Bcl-2 or Bcl-x_L prevents cells entering into apoptosis, but cells remain arrested in G_2-M. Inhibition of caspases also inhibits apoptosis. Two phases can be separated in the treatment of HL-60 cells with MTC: a reversible phase in which microtubules can be reassembled and the normal cell cycle can be restored after MTC removal; and an irreversible phase in which the triggering of apoptosis has occurred and cells are committed to die. For further details, see the text.

(40). Overall, our data indicate that cells can be deprived of cytoplasmic microtubules and arrested in G_2-M for a rather prolonged period of time before the irreversible apoptotic signaling is triggered. The length of this lag time could reflect the balance between apoptotic/survival signals in a particular cell type. JNK activation is detected as an early event in MTC-treated cells during the MTC reversible phase. Later on, c-*jun* induction is also detected during the reversible phase. Because the initiation of these MTC-mediated processes occur during the period where MTC actions are reversible (Fig. 8), this suggests that triggering of these two processes is not sufficient to induce the irreversible onset of apoptosis. Removal of MTC, after inducing JNK activation and c-*jun* expression but not DNA degradation, prevented any apoptotic response. However, although MTC-induced JNK activation and c-*jun* expression were initiated during the MTC reversible phase, these two responses were persistent and were maintained beyond the end of the reversible phase. Thus, their sustained activation could lead to an accumulative effect that ultimately leads to the induction of apoptosis. The data reported here highlight the importance of timing and duration of the responses elicited by MTC to achieve a final outcome, separating reversible and irreversible processes, and lead to the notion that a threshold for c-*jun* expression and JNK activation, which in turn stimulates c-*jun* transcription, should be achieved to raise the irreversible triggering of apoptosis. This seems to occur for other antitumor agents, such as the ether lipid ET-18-OCH_3 (29, 32). Sustained induction of c-*jun* expression and JNK activation have been involved in the apoptotic responses to distinct agents (32, 33–37). This indicates that the activation of certain signaling routes putatively involved in apoptosis do not constitute by themselves an irreversible process, and a threshold must be reached before committing cells to an irreversible apoptotic response. Our data on JNK activation are in agreement with a previous report (41) showing JNK activation by distinct microtubule-interfering agents as a putative stress response to the disruption of microtubule dynamics. MTC also induces caspase activation assayed by the cleavage of the typical caspase-3 substrate PARP, as a late event in the cascade of biochemical processes turned on by MTC in the irreversible phase. The use of a broad caspase inhibitor, z-Asp-DBMC, prevented PARP cleavage and protected cells from apoptosis in MTC-treated cells, indicating the contribution of caspases to the execution of MTC-induced apoptosis.

Because HL-60 cells lack p53 expression (42), these results indicate that MTC-induced apoptosis is not dependent on this tumor suppressor protein. This is in agreement with previous data demonstrating that inactivation of p53 does not confer resistance against Taxol-induced apoptosis (43).

Overall, MTC is a bicyclic colchicine analogue that binds rapidly and reversibly to the high affinity colchicine binding site of the tubulin molecule, inhibiting microtubule assembly. As a consequence, it blocks the cell cycle at G_2-M, induces JNK activation and c-*jun* expression in a persistent way, promotes caspase activation, and induces apoptosis in human leukemic cells. A remarkable feature of this compound is its reversible effect on microtubule disassembly and G_2-M cell cycle arrest. These results suggest that MTC can show a lower toxicity than colchicine and other microtubule-interfering agents. In addition, unlike other drugs acting on microtubules, including Taxol, MTC is water-soluble.

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