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-XL and Cell Cycle Arrest

Consuelo Gajate, Isabel Barasoain, José M. Andreu, and Faustino Mollinedo

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 G2-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 G2-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 NH2-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-xI, by gene transfer in human erythroleukemic HEL cells abrogated MTC-induced apoptosis, but cells remained arrested in G2-M, suggesting that bcl-2 and bcl-xI block the signaling pathway between G2-M arrest and triggering of apoptosis. MTC-treated bcl-2 and bcl-xI-transfected HEL cells recovered their capacity to proliferate after MTC removal. These results indicate that microtubule inhibition induces G2-M arrest and apoptosis in leukemia cells, showing a lag phase between G2-M arrest and the onset of apoptosis, regulated by bcl-2 and bcl-xI, during which MTC displays a reversible action on microtubule depolymerization and G2-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. 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. Paclitaxel binds and stabilizes microtubules, thereby suppressing their dynamics. This results in G2-M arrest, microtubule bundling, and cell death. 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 alkaldoid 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. Interaction of colchicine with tubulin is attributable to the simultaneous binding of its trimethoxyphenyl A and 2-methoxypropenoic 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 b-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 MTC3 (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-methoxypropenoic 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
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-methoxypropene 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 G2-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 G2-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. Gorbanoff (Brandeis University). Colchicine was purchased from Aldrich Chemical Co. (St. Louis, MO). Lumicolchicine was prepared by long wavelength UV irradiation of colchicine. RPMI 1640 culture medium, FCS, antibiotics, and L-glutamine (Steinheim, Germany). Paclitaxel (Taxol) was from Sigma Chemical Co. (St. Louis, MO). Colchicine was purchased from Aldrich Chemical Co. (St. Louis, MO). MTPC was kindly provided by Dr. M. Gorbunoff (Cambridge, MA). Rabbit anti-Bcl-xL antiserum was from Transduction Laboratories (Lexington, Kentucky). The caspase inhibitor z-Asp-DBMC (5 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 (2000 g, 5 min) 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-ethanesulfonic acid), 1 mM EGTA, and 1 mM MgCl₂ (pH 6.8)] for 30 min, and immunofluorescence was performed with DM1A monoclonal antibody reacting with α tubulin and fluorescein-labeled goat anti-mouse immunoglobulin as described previously (26). Cytoskeletons were observed with a Zeiss Axiosplan 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-G1 peak in cell cycle analysis (28). Briefly, cells (5 × 10⁶) 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.

[^3H]Thymidine Incorporation. HL-60 cells (1.25 × 10⁶ 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 [^3H]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 [^3H]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 ≥7 × 10⁶ cpm/μg of cDNA. cDNA probe for c-jun (30) was kindly provided by Dr. R. Bravo (Quibb 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 × 10⁶) were resuspended in 200 μl of extract buffer (25 mM HEPES (pH 7.7), 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na₂VO₄, 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.

Fig. 1. Chemical structures of colchicine, lumicolchicine, MTC, and MTPC.
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 MgCl₂, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 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 was 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 MgCl₂, 0.1 mM Na₃VO₄, 20 μM ATP] and incubated with 4 μCi [γ-32P]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 NH₂-terminal domain (31).

**Western Blot Analysis.** About 6 × 10⁶ 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% powdered defatted milk, and incubated with mouse monoclonal anti-PARP antibody, mouse monoclonal anti-Bcl-2, or rabbit anti-Bcl-xL antisemur. 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. 2A). 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 [3H]thymidine incorporation (Fig. 2A). 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. 2B). 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. 2A) or apoptosis (Fig. 2B). 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. 2C) 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...
microtubule disruption and on cell cycle arrest, whereas colchicine (data not shown). Thus, we found a reversible action of MTC on not allow cell cycle reversion, and cells were directed to apoptosis MTC removal (data not shown). In contrast, colchicine removal did underwent a strong apoptotic response (Fig. 4). A partial restoration of the microtubule network and appearance of mitosis in the case of MTC-treated cells (f), whereas the colchicine effect was not reversed (E). Bar, 10 μm. The photomicrographs shown are representative of at least three independent experiments performed.

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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. 4A). After 30–60 min of incubation, MTC-treated cells started to accumulate in G2-M, reaching a maximum after 6–9 h of incubation, with a parallel decrease in cells in G0/G1 (Fig. 4A). The appearance of cells with a DNA content less than G1, characteristic of early apoptotic cells (sub-G1 peak; Fig. 4A), could be observed after 9 h of treatment with 1 μM MTC together with a significant decrease in the proportion of cells in G2-M (Fig. 4A). At 24 h of incubation with 1 μM MTC, about 66% of the cells underwent apoptosis (Fig. 4A). 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 G2-M without a significant induction of apoptosis (Fig. 4A). 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 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. 4C). 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. 4C). This indicates that the cell cycle arrest in G2-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. 5A). Colchicine also induced a significant increase in c-jun expression (Fig. 5A). 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. 5A) 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.
Control), treated with 1 \(\mu\)M MTC for 6 and 24 h, and HL-60 cells treated with 1 \(\mu\)M 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 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 \(\mu\)M MTC for 6 and 24 h, and HL-60 cells treated with 1 \(\mu\)M 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 shown are representative of four independent experiments performed.}

**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 \(\mu\)M 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 \(\mu\)M MTC for 6 and 24 h, and HL-60 cells treated with 1 \(\mu\)M 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 shown are representative of four independent experiments performed. C, reversible effect of MTC on cell proliferation. HL-60 cells (1.8 \(\times\) 10^6) were grown for 24 h in the absence of any drug (Control), treated with 1 \(\mu\)M MTC for 24 h, or treated with 1 \(\mu\)M 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 \(\pm\) SD.

**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 \(\mu\)M colchicine or 1 \(\mu\)M MTC for the times indicated. Basal control levels in untreated HL-60 cells are also shown. RNA 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 \(\mu\)M 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.
of Bcl-2 (Fig. 7C) and Bcl-xL (data not shown) did not affect the cell cycle arrest in G2-M phase induced by MTC. As shown in Fig. 7C, bcl-2-transfected HEL cells accumulated in the G2-M phase upon MTC treatment, and after 24 h of treatment, practically the whole cell population was arrested in the G2-M phase. Bcl-2-transfected HEL cells remained arrested in G2-M for prolonged periods of time (at least 48 h) without undergoing apoptosis (Fig. 7B). Thus, overexpression of Bcl-2 completely prevented MTC-induced apoptosis, but did not affect the cell cycle arrest in G2-M promoted by MTC. Similar results concerning prevention of apoptosis and arrest in G2-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-xL-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. 7D). 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, G2-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-xL 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 G2-M, reaching a significant G2-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-xL efficiently inhibited apoptosis induced by MTC as well as by other microtubule-
active agents, such as colchicine and Taxol, without affecting their actions on the cell cycle, i.e., G2-M arrest. This indicates that G2-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-xL-transfected cells can continue to proliferate after MTC removal. Putative mechanisms by which Bcl-2 and Bcl-xL interfere with signaling between G2-M arrest and apoptosis onset include prevention of mitochondrial transmembrane potential collapse, cytochrome c release, and caspase-9 activation.
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, G2-M arrest, and induction of apoptosis) are placed in boxes. Overexpression of Bcl-2 or Bcl-xL prevents cells entering into apoptosis, but cells remain arrested in G2-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.

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 G2-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 G2-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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