Apoptotic Response of HL-60 Human Leukemia Cells to the Antitumor Drug TAS-103

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ABSTRACT

TAS-103 is a DNA intercalating indeno-quinoline derivative that stimulates DNA cleavage by topoisomerases. This synthetic drug has a broad spectrum of antitumor activity against many human solid tumor xenografts and is currently undergoing clinical trials. We investigated the induction of apoptosis in human promyelocytic leukemia cells treated with TAS-103. The treatment of proliferating human leukemia cells for 24 h with various concentrations of the drug produces significant variations in the mitochondrial transmembrane potential (ΔΨmt) measured by flow cytometry using the fluorochromes 3,3′,diethyloxacarbocyanine iodide, Mitotracker Red, and tetrachloro-tetraethylbenzimidazolocarbocyanine iodide. The collapse of ΔΨmt is accompanied by a marked decrease of the intracellular pH. Cleavage experiments with the substrates N-acetyl-Asp-Glu-Val-Asp-pNA, poly(ADP-ribose) polymerase, and pro-caspase-3 reveals unambiguously that caspase-3 is a key mediator of the apoptotic pathway induced by TAS-103. Caspase-8 is also cleaved, and the bcl-2 oncoprotein is underexpressed. Drug-induced internucleosomal DNA fragmentation and the externalization of phosphatidylserine residues in the outer leaflet of the plasma membrane were also characterized. The cell cycle perturbations produced by TAS-103 can be connected with the changes in ΔΨmt. At low concentrations (2–25 nM), the drug induces a marked G2 arrest and concomitantly provokes an increase in the potential of mitochondrial membranes. In contrast, treatment of the HL-60 cells with higher drug concentrations (50 nM to 1 μM) triggers massive apoptosis and a collapse of ΔΨmt that is a signature for the opening of the mitochondrial permeability transition pores. The discovery of a correlation between the G2 arrest and changes in mitochondrial membrane potential provides an important mechanistic insight into the action of TAS-103.

INTRODUCTION

TAS-103 (Fig. 1) is an indeno-quinoline derivative that exhibits a broad spectrum of antitumor activity against many human solid tumor xenografts including cancer of the lung, pancreas, and kidney (1–3). This synthetic drug has shown synergistic effects with cisplatinum to reduce the growth of SBC-3 cells, suggesting that the association of cisplatinum and TAS-103 may be useful for the treatment of small cell lung cancer (4). At the molecular level, TAS-103 is a DNA-intercalating agent capable of stimulating DNA cleavage by mammalian topoisomerase I and human topoisomerases IIα and IIβ in vitro. However, recent studies indicate that topoisomerase II is the primary cellular target of TAS-103 (5–7). The study with a yeast genetic system (6) concludes that TAS-103 should now be classified as a topoisomerase II-targeted drug rather than a dual topoisomerase I/II poison, as was initially thought.

Topoisomerase inhibition generally results in cell cycle arrests and/or activation of the apoptotic pathway. In the present study, we examine the effects of TAS-103 on HL-60 cells, a human promyelocytic cell line. These leukemia cells are prone to enter apoptosis on treatment with a variety of stimuli including topoisomerase inhibitors, such as camptothecin and etoposide (8–10). Specifically, we were interested in characterizing a variety of apoptotic events at the mitochondrial and nuclear levels. We show that TAS-103 induces a collapse of the mitochondrial transmembrane potential and a marked decrease in intracellular pH. The drug activates caspase-3 and induces internucleosomal DNA fragmentation and externalization of PS3 residues. Interestingly, we found a relation between the effects of the drug on cell cycle distribution and the modification of mitochondrial transmembrane potential. The study identifies and delineates signaling factors involved in TAS-103-induced apoptosis in HL-60 cells.

MATERIALS AND METHODS

Drugs and Chemicals

TAS-103 was provided to one of us [N. O.] by Taiho Chemicals Co. (Tokyo, Japan). Etoposide, camptothecin, oligomycin, and staurosporine were from Sigma Chemical Co. (La Verpillière, France). Nigericin, DiOC6(3), chloromethyl-X-rosamine (Mitotracker Red), JC-1, SNARF-AM, and carbonyl cyanide p-chlorophenylhydrazone were from Molecular Probes (Eugene, OR). All other chemicals were analytical-grade reagents.

Cell Cultures and Survival Assay

Human HL-60 promyelocytic leukemia cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C in a humidified atmosphere containing 5% CO2 in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). The cytotoxicity of TAS-103 was assessed using a cell proliferation assay developed by Promega (CellTiter 96 AQuescent One Solution Cell Proliferation Assay). Briefly, 2 × 104 exponentially growing cells were seeded in 96-well microculture plates with various drug concentrations in a volume of 100 μl. After a 72-h incubation at 37°C, 20 μl of MTS were added to each well, and the samples were incubated for a further 3 h at 37°C. Plates were analyzed on a Labsystems Multiskan MS type 352 reader at 942 nm. The live/dead fluorometric assay was performed according to the supplier’s recommended protocol (Molecular Probes). In this case, the flow cytometry analysis was done using FI-1 (530 nm, log scale) for calcein-AM and FI-3 (620 nm, linear scale) for EthD-1.

Cell Cycle Analysis

For flow cytometry analysis of DNA content, 105 HL-60 cells in exponential growth were treated with graded concentrations of TAS-103 for 24 h and then washed three times with citrate buffer. The cell pellet was incubated with 250 μl of trypsin-containing citrate buffer for 10 min at room temperature and then incubated with 200 μl of citrate buffer containing a trypsin inhibitor and RNase (10 min) before adding 200 μl of PI at 125 μg/ml. Samples were analyzed on a Becton Dickinson FACScan flow cytometer using LSYS II software, which is also used to determine the percentage of cells in the different phases of the cell cycle. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm (FL-3).

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3 The abbreviations used are: PS, phosphatidylserine; DioC6(3), 3,3′,diethyloxacarbocyanine iodide; JC-1, tetrachloro-tetraethylbenzimidazolocarbocyanine iodide; PARP, poly(ADP-ribose) polymerase; DEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-pNA; SNARF-AM, carbonyl cyanide-SNARF-1-acetoxymethyl ester; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; EthD-1, ethidium homodimer-1; AM, acetoxyethyl ester; PBST, 0.1% Tween 20 and 25 mM phosphate buffer (pH 7.4).
incubation in a CO₂ incubator at 37°C, cells were pelleted, rinsed once with HBSS before adding 20 μl of carboxy-SNARF-AM at 10 μM. After a 1-h incubation in a CO₂ incubator at 37°C, cells were pelleted, rinsed once with HBSS, and resuspended at an appropriate density for fluorescence measurements. The fluorescence excitation was set up at 488 nm, and the emission was recorded at 575 and 620 nm. Intracellular pH was estimated by comparison of the mean ratio values (fluorescence at 575 nm divided by fluorescence at 620 nm) of a sample to a calibration curve established by incubation of SNARF-AM-loaded cells in varied pH buffer in the presence of the proton ionophore nigericin.

DNA Fragmentation

HL-60 cells at a density of 5 × 10⁵ cells/ml were treated with various concentrations of TAS-103 for the indicated periods and then collected by centrifugation at 2500 × g for 5 min. The resulting cell pellets were resuspended in PBS containing 5 mM MgCl₂ and lysed in 500 μl of Tris-EDTA buffer containing 0.1% SDS and proteinase K (1.5 mg/ml) overnight at 37°C. After two successive extractions with phenol/chloroform, the aqueous layer was transferred to a new centrifuge tube. The DNA was precipitated with ethanol, resuspended in water (100 μl), and treated with RNase A (100 μg/ml) for 2 h at 37°C. Electrophoresis was performed in 1% agarose gel in Tris-borate buffer at about 12 V/cm for approximately 4 h. After electrophoresis, the gel was stained with ethidium bromide (1 mg/ml), washed, and photographed under UV light.

DEVD-pNA Cleavage

DEVD-pNA cleavage activity was measured using the ApoAlert CPP32/caspase-3 assay kit (Clontech, Palo Alto, CA), and the recommended protocol was followed. Briefly, 2 × 10⁶ exponentially growing HL-60 cells in 2 ml of RPMI 1640 were treated with the test drug at the indicated concentration for 24 h at 37°C. Cells were pelleted by centrifugation and resuspended in 50 μl of the lysis buffer. The lysed cell mixture was then incubated on ice for 10 min before centrifugation (18,300 × g, 3 min at 4°C). Fifty μl of 2× reaction buffer supplemented with 10 mM DTT were then added to each tube incubated at 4°C. During this period, a control was prepared by adding 0.5 μl of 1 mM DEVD-fmk to a cell sample treated with 0.1 μM staurosporine (24 h at 37°C). The substrate DEVD-pNA was added to all tubes (5 μl, 50 μM), and the samples were incubated for 1 h at 37°C. The formation of p-nitroanilide was measured at 405 nm using a Labsystems Multiskan MS microtiter plate reader.

Western Blotting

PARP Cleavage. Briefly, 7 × 10⁶ exponentially growing HL-60 cells in a serum-free medium were treated with the test drug at the indicated concentration for 24 h at 37°C. Cells were pelleted by centrifugation, and resuspended in 3 mL of lysis buffer containing 25 mM PBS, 0.1 mM phenylmethylsulfonyl fluoride, and the protease inhibitors chymostatin, leupeptin, aprotinin, and pepstatin A (5 μg/ml each). After centrifugation, the pellet was resuspended in the loading buffer containing 50 mM Tris-HCl (pH 6.8), 1% sucrose, 2 mM EDTA, 3% SDS, and 0.01% bromphenol blue. The mixture was sonicated for 30 s at 4°C and then boiled to 100°C for 3 min. For Western blotting, the cell lysates were fractionated on a 7.5% polyacrylamide gel containing 0.1% SDS and then transferred onto a Hybond-C nitrocellulose membranes (Amersham) for 40 min at 0.8 mA/cm² using a semidry transfer system. Membranes were blocked with 10% nonfat milk in PBST for 30 min, followed by incubation with anti-PARP monoclonal antibody (Clontech; 1:10,000 dilution in PBST supplemented with 0.1% nonfat milk) for 30 min. The blots were washed three times (5 min each with PBST) and incubated with a horseradish peroxidase-conjugated goat antimouse IgG (Amersham Life Sciences; 1:10,000 dilution in PBST containing 0.1% nonfat milk) for 30 min. After three successive washes with PBST, the Western blot chemiluminescence reagent from New England Nuclear (Boston, MA) was used for detection. Bands were visualized by autoradiography.

Pro-Caspase-3 Processing, Caspase-8 Activation, and Decreased bcl-2 Expression. HL-60 cells (0.7 × 10⁶ in 1 ml) were treated with TAS-103 at the indicated concentration for 24 h at 37°C. Cells were pelleted by centrifugation at 4°C and washed twice with PBS (2 × 3 ml) at 4°C. After centrifugation, the pellet was resuspended in 25 μl of boiling buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM sodium vanadate, 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and the protease inhibitors leupeptin (5 μg/ml), aprotinin (10 μg/ml), and pepstatin A (2.5 μg/ml). The mixture was incubated for 10 min at 4°C before adding 75 μl of the electrophoresis dye solution (15% sucrose, 50 mM Tris-HCl, 2 mM EDTA, 3% SDS, and 0.01% bromphenol blue). Samples were passed through a 26-gauge needle to reduce the viscosity of the solutions and then boiled to 100°C for 3 min. For Western blotting, the cell lysates (containing about 30 μg of proteins) were fractionated on a 12.5% polyacrylamide gel containing 0.1% SDS and then transferred onto Hybond-C nitrocellulose membranes (Amersham) for 40 min at 0.8 mA/cm² using a semidry transfer system. Membranes were blocked with 10% nonfat milk in PBST for 1 h at room temperature (or overnight at 4°C), followed by incubation with a mouse monoclonal antibody directed against bcl-2 (1:1,000; Immunotech), caspase-8 (1:1,000; Immunotech), or actin (1:1,000; Oncogene Research Products). A rabbit polyclonal antibody was used to detect pro-caspase-3 (1:1,000; PharMingen). In all cases, antibodies were diluted in PBST containing 2% nonfat milk, and membranes were incubated for 4 h in the dark with gentle agitation. The blots were washed three times (15 min each) with PBST and incubated with a sheep antiperoxidase or antirabbit IgG conjugated to horseradish peroxidase (Amersham Life Sciences; 1:10,000 dilution in PBST containing 2% nonfat milk) for 1 h. After three successive washes (15 min each) with PBST, the Western blot chemiluminescence reagent from New England Nuclear was used for detection.

TUNEL Assay. The Apoptosis Detection System, Fluorochrome developed by Promega was used according to the supplier’s recommended protocol. Briefly, after the drug treatment, 5 × 10⁶ cells were centrifuged, washed twice with PBS, and gently resuspended in 0.5 ml of PBS before adding 5 ml of 1% ice-cold paraformaldehyde for 20 min. Fixed cells were washed with 5 ml of PBS and resuspended in 0.5 ml of PBS and 5 ml of cold 70% ethanol. Dehydrated cells were then incubated for 4 h at −20°C. The cells were washed again with 5 ml of PBS and finally transferred to a 1.5-ml microfuge tube and centrifuged for 10 min at 20°C. The supernatant was discarded, and the pellet was resuspended in 80 μl of equilibration buffer for 5 min at room temperature. After another round of centrifugation, the nuclei were incubated for 1 h at 37°C in the dark in 50 μl of equilibration buffer containing fluorescent 12-deoxyuridine-5'-triphosphate (dUTP) in the presence of terminal deoxynucleotidyl transferase to label 3'-OH ends of fragmented DNA. The reaction was stopped by adding 1 ml of 20 mM EDTA with gentle stirring. After centrifugation, the material was resuspended
in PBS containing 0.1% Triton X-100 and 5 mg/ml BSA. After a second wash, the material was resuspended in 0.5 ml of PBS containing 5 μg/ml PI and 250 μg of RNase A. The mixture was incubated at room temperature in the dark for 30 min before analysis by flow cytometry for both DNA breaks (TUNEL) using FI-1 (575 nm, log scale) and DNA content (PI) using FI-3 (620 nm, linear scale).

**RESULTS**

**Cytotoxicity.** TAS-103 is highly toxic to HL-60 cells. Using a conventional tetrazolium-based MTS assay (11), we evaluated the IC_{50} to 40 nM after a 72-h incubation period. The cytotoxic potential of TAS-103 toward HL-60 cells was investigated further using a double-labeling procedure with the fluorescence markers calcein-AM and EthD-1, which simultaneously stain live and dead cells in green and red, respectively (Fig. 2). Intracellular esterases in live cells can convert the virtually nonfluorescent cell-permeable calcein-AM into the intensely fluorescent calcein. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence on binding to DNA, thereby producing a bright red fluorescence in dead cells. Cells were treated with graded concentrations of TAS-103 for 24 h and then loaded with calcein-AM and EthD-1 before analysis by flow cytometry. The populations of live (calcein-positive) and dead (EthD-1-positive) cells can be easily differentiated; however, in addition, a third population corresponding to cells stained with both calcein and EthD-1 can be detected. This double-stained cell fraction represents 21% of the cells on treatment with 50 nM TAS-103 and reaches 34% with 0.5 μM drug. Cells with an active metabolism (calcein positive) that allow EthD-1 to penetrate and stain their nucleic acids most likely correspond to apoptotic cells. This fluorescence activation/dye exclusion (live/dead) assay may prove useful to quantify drug-induced apoptotic cell death.

**Cell Cycle Effects.** Treatment of HL-60 cells with increasing concentrations of TAS-103 for 24 h led to profound changes of the cell cycle profiles (Fig. 3). The flow cytometry analysis of PI-labeled cells indicates that treatment with low drug concentrations (2–25 nM) induces a massive accumulation of cells in the G2-M phase. The G2 cell population increases from 16% in the control to 60% in the presence of 10 nM TAS-103. In the mean time, the G1- and S-phase cell populations gradually decrease from 46% and 35% to 13% and 18%, respectively. In contrast, with higher drug concentrations (50–500 nM), the G2-M-phase fraction disappears. Apoptosis appears with concentrations of >20 nM. A characteristic hypodiploid DNA content peak (sub-G1) can be detected with 25 nM TAS-103 and is maximal at 50 nM. The induction of apoptosis is cell cycle dependent. The decrease in the G2-M-phase cell population observed with concentrations of >20 nM could be explained by a decrease in the S-phase cell entrance in G2 phase due to apoptosis. The accumulation of topoisomerase-mediated DNA strand breaks likely causes the failure of DNA repair and the subsequent inactivation of the G2 checkpoint mechanism and concomitantly switches on the cell death signal. It is likely that several proteins (e.g., cyclin B and p34^{cdk2}) participating in the G2 checkpoint mechanism that regulates normal cell cycle progression are activated by TAS-103. Both the live/dead assay and the cell cycle analysis suggest that myeloid leukemia HL-60 cells accomplish apoptosis on TAS-103 treatment. Therefore, we examined a variety of apoptotic events using several complementary cytometric and biochemicals methods.

**Variations of the Mitochondrial Membrane Potential.** Mitochondria plays an essential role in the propagation of apoptosis. It is well established that at an early stage, apoptotic stimuli alter the mitochondrial transmembrane potential (ΔΨ_m) (12). We first used the ampholytic cationic fluorochrome DiOC_6 to monitor the changes in ΔΨ_m induced by TAS-103. HL-60 cells were treated with graded concentrations of the drug (2 nM to 1 μM) for 24 h and then analyzed by flow cytometry after DiOC_6 labeling. The results presented in Fig. 4A. Cells treated with concentrations of >30 nM exhibit a significant reduction in cellular uptake of the fluorochrome. The decrease of fluorescence intensity measured with the DiOC_6 probe reflects the collapse of ΔΨ_m, which is a signature for the opening of the mitochondrial megachannels, also called the permeability transition pores (13). In contrast, we observe an increase in DiOC_6 fluorescence when the cells were treated with low concentrations of TAS-103 (<30 nM). It is important to mention that the hyperpolarization effect detected on treatment with low concentrations of TAS-103 was always observed using either 25, 50, or 100 nM DiOC_6.

The dissipation of ΔΨ_m observed on treatment with drug concentrations of >30 nM is characteristic of apoptosis and has been commonly observed with a variety of anticancer drugs, irrespective of the cell type. The disruption of the ΔΨ_m generally defines an early but already irreversible stage of apoptosis (12). Conversely, the fluorescence enhancement observed on treatment with low drug concentra-

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Fig. 2. Three-dimensional representation of the correlated distribution of calcein and EthD-1 fluorescence in HL-60 cells treated with graded concentrations of TAS-103 for 24 h.
brane functionality may contribute to the observed increase in DiOC6 fluorescence. Side effects due to a small modification of the plasma membrane and can partially anchor into the plasma membrane with an increase in DiOC6 fluorescence has been reported in a study of acetylceramide-induced apoptosis of monoblastoid U937 cells (16). It has been known for a long time that mitochondrial metabolic changes are associated with changes in mitochondrial volume. In the absence of any change in membrane potential, mitochondrial swelling by itself should induce an increase in the matrix accumulation of membrane potential probe DiOC6 and thus induce an increase in fluorescence. Such a mitochondrial swelling with an increase in DiOC6 fluorescence has been reported in a study of acetylceramide-induced apoptosis of monoblastoid U937 cells (16). It has been known for a long time that mitochondrial metabolic changes are associated with changes in mitochondrial volume (including both swelling and contraction (17, 18)). A second hypothesis can be offered: DiOC6 is not entirely specific for mitochondrial membranes and can partially anchor into the plasma membrane. Side effects due to a small modification of the plasma membrane functionality may contribute to the observed increase in DiOC6 fluorescence. However, this second hypothesis is unlikely because we observed the same effects (hyperpolarization followed by depolarization at higher drug concentrations) using the fluorescence probes Mitotracker Red (Fig. 4B) and JC-1 (Fig. 4C), which are both more specific to mitochondrial membranes than DiOC6. JC-1 has the unique property of forming red-fluorescent J-aggregates locally and spontaneously under high mitochondrial ∆Ψmt whereas the monomeric form fluoresces in green (19). The changes in the ratio of red:green fluorescence reflect the variations of ∆Ψmt. Untreated cells simultaneously exhibit intensive green and red fluorescence. Apoptotic HL-60 cells obtained by treatment with 100 nM TAS-103 exhibit an extinction of red fluorescence. In contrast, an increase in green fluorescence was observed with the G2-blocked cells receiving 25 nM TAS-103. The experiments using Mitotracker Red and JC-1 extend the results described above with DiOC6 and confirm unambiguously that TAS-103 first induces an increase at low concentrations, followed by a marked decrease of ∆Ψmt at higher drug concentrations.

**pH Changes.** Intracellular acidification is a relatively early and common feature of the apoptotic program (20). For example, previous studies have shown that topoisomerase inhibitors etoposide and camptothecin lower the pH value of HL-60 cells (21, 22). By analogy, we reasoned that TAS-103 might also affect the intracellular pH, and this could contribute to the propagation and/or amplification of apoptosis. Treated and control cells were loaded with the pH-sensitive dye carboxy-SNARF-1-AM, and the pH in individual cells was determined using ratiometric flow cytometry. Excitation was set up at 488 nm, and fluorescence emission was monitored at 575 and 620 nm. Fig. 5 shows that the intracellular pH drops significantly from 7.3 to up to 6.2 on treatment with TAS-103 (0.5 μM). Treatment with a 10-fold lower drug concentration, 0.05 μM, enabled us to distinguish the normal and apoptotic cell population with a low intracellular pH.

**Caspase Activation.** Caspases are at the heart of the apoptotic machinery (23). Several caspases have been shown to be key executors of apoptosis mediated by various inducers, including antitumor agents (24). For example, caspase-3, which cleaves DEVD-type substrates, is involved in camptothecin-induced apoptosis in HL-60 cells (10). It was therefore of interest to determine whether this cysteine protease is also involved in the apoptosis induction by TAS-103 in HL-60 cells. An initial way to address this question is through the use of the caspase-3 peptide substrate DEVD-pNA. We prepared lysates from cells treated for 24 h with various concentrations of the drug and then assayed for an activity capable of cleaving DEVD-pNA using a solution assay. Lysates were mixed with the pNA-tagged tetrapeptide, and the absorbance of the released substrate was recorded at 405 nm using the 96-well plate reader. A marked activity was recorded in

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**Fig. 3.** Cell cycle analysis of HL-60 cells treated with graded concentrations of TAS-103 for 24 h. Cells were analyzed using the FACScan flow cytometer as described in “Materials and Methods.”

**Fig. 4.** Mitochondrial functional changes as a consequence of TAS-103 treatment. The cytofluorometric profiles show hyperpolarization with low drug concentrations (2–25 nM), followed by hyperpolarization observed with higher concentrations of TAS-103. Cells were stained with the ∆Ψmt-sensitive dye (A) DiOC6 (100 nM), (B) Mitotracker Red (1 μM), and (C) JC-1 (2 μg/ml) before analysis by flow cytometry.
lysates from cells treated with TAS-103 and with staurosporine, etoposide and camptothecin but no activity was apparent in the control (drug-free) lysates (Fig. 6). However, DEVD may not be cut exclusively by caspase-3. It may also be a substrate for caspase-7. A recent study of inhibitor specificity found that z-DEVD-fmk inhibits both caspase-3 and -7 (25). Therefore, we used a second method based on the cleavage of PARP, an enzyme involved in DNA repair that has been shown to serve as a substrate for caspase-3 (26).

PARP catalyzes the transfer of the ADP ribose moiety from its substrate, NAD$^+$, to a limited number of protein acceptors involved in chromatin architecture or in DNA metabolism, including topoisomerase I (27, 28). The Western blot analysis in Fig. 7a demonstrates that the $M_1$ 116,000 PARP protein was cleaved into its characteristic $M_1$ 89,000 fragment on treatment of the cells with TAS-103. The $M_1$ 24,000 fragment is not detected by the anti-PARP antibody that recognizes only the COOH terminus of the protein. With both TAS-103 and camptothecin, a treatment for 24 h with 0.1 $\mu$m drug suffices to induce quantitative cleavage of PARP in the leukemia cells. Shorter treatments (for 4 and 6 h) with 0.1 $\mu$m drug caused about 20% and

Fig. 5. Intracellular acidification of HL-60 cells treated with TAS-103. Cells were incubated with the drug at the indicated concentration for 24 h before loading with 1 $\mu$m SNARF-AM for 1 h and fluorescence analysis by flow cytometry. The fluorescence excitation was set up at 488 nm, and the emission was recorded at 575 and 620 nm. The indicated pH values are derived from the average fluorescence ratio for the whole cell population, as determined from calibration curves.

Fig. 6. A, TAS-103 promotes DEVD-pNA cleaving activity. HL-60 cells were incubated with the test drug at the indicated concentration for 24 h before adding the caspase-3 substrate DEVD-pNA (50 $\mu$m). Assay mixtures were incubated for 1 h at 37°C before measurement of the absorbance at 405 nm. The histogram in B shows DEVDase activity without drug (control, Ct) and in the presence of 0.1 $\mu$m staurosporine (St), 0.05 $\mu$m TAS-103 (TAS), 1 $\mu$m camptothecin (CPT), or 0.1 $\mu$m staurosporine plus the peptidic inhibitor of caspase-3 DEVD-fmk at 5 $\mu$m (+Inh.). Results are the mean of three experiments.

Fig. 7. Western blot analysis for the cleavage of (a) PARP, (b) procaspase-3, and (c) caspase-8, and (d) the expression of bcl-2. Control lanes (Ct) refer to untreated cells. In the other lanes, the cells were treated with TAS-103 at the indicated concentration ($\mu$m) for 24 h. In each case, whole cell lysates were subjected to SDS-PAGE followed by blotting with an anti-PARP, anti-procaspase-3, anti-caspase-8, or anti-bcl-2 monoclonal antibody. Camptothecin (CPT) and etoposide (Etop.) were used at 0.1 and 1 $\mu$m, respectively. Arrows point to the full-length proteins. The molecular weight of each protein is indicated. In c and d, the $M_1$ 42,000 band refers to actin that was detected with an anti-actin monoclonal antibody to confirm an equal amount of protein in each lane.
activation of pro-caspase-3 (Fig. 7) and the intracellular levels of the irreversible apoptotic pathway. too frequent/abundant to be repaired, and therefore the cells engage in (sub-G₁) starts to appear in the cell cycle experiments. There is little nM), which cause a profound G₂ arrest. Therefore, we are inclined to or no cleavage of PARP with low drug concentrations (range, 2–20 nM, the damages caused to the genetic material in the HL-60 cells are unable to cope with a saturating DNA injury arising from the topoisomerase-directed genotoxic insult. With drug concentrations of ≥25 nM, the hypodiploid peak (sub-G₁) starts to appear in the cell cycle experiments. There is little or no cleavage of PARP with low drug concentrations (range, 2–20 nM), which cause a profound G₂ arrest. Therefore, we are inclined to believe that the cleavage of PARP might be a sign that the cell is unable to cope with a saturating DNA injury arising from the topoisomerase-directed genotoxic insult. With drug concentrations of ≥25 nM, the damages caused to the genetic material in the HL-60 cells are too frequent/abundant to be repaired, and therefore the cells engage in the irreversible apoptotic pathway.

In parallel, we followed by immunoblot analysis the proteolytic activation of pro-caspase-3 (Fig. 7b) and the intracellular levels of caspase-8 (Fig. 7c) in HL-60 cells treated with TAS-103. A distal caspase such as caspase-3 can be directly activated by a proximal caspase such as caspase-8 (29, 30). Caspase-8 is synthesized as two isoforms of Mr ~55,000. In both cases, a concentration of 50 nM TAS-103 was sufficient to induce quantitative cleavage of pro-caspase-3 and caspase-8 after a 24-h incubation period. These experiments indicate that TAS-103 activates both distal and proximal caspasess. The activation of caspase-8 shown by the Western blot experiments in Fig. 7c was independently confirmed by colorimetry using the N-acetyl-Ile-Glu-Thr-Asp-pNA peptide substrate (data not shown).

Reduced Expression of bcl-2. The bcl-2 oncoprotein located on the outer mitochondrial membrane is important for the suppression of apoptosis and mitochondrial manifestations of apoptosis (31). bcl-2 prevents the initiation of the cellular apoptotic program by stabilizing the mitochondrial permeability transition and avoiding the subsequent release of cytochrome c to prevent caspase activation (32). In doing so, the protein can block apoptotic death in multiple contexts, including the case of topoisomerase II inhibitors (33). The immunoblot analysis presented in Fig. 7d indicates that treatment of the cells with TAS-103 induces a down-regulation of bcl-2. Low drug concentrations (25 nM) suffice to considerably reduce the level of bcl-2 proteins in HL-60 cells. This effect must facilitate caspase activation.

Externalization of PSs. PS lipids are normally confined to the inner leaflet of the plasma membrane but are exported to the outer plasma membrane leaflet during apoptosis to serve as a trigger for recognition of apoptotic cells by phagocytes (34). PS can be detected by staining with a FITC conjugate of annexin V, a Mr 38,000 protein that binds naturally to PS (35). HL-60 cells treated with TAS-103 for 24 h were found to be positive for PS in the outer leaflet (Fig. 8). Approximately 55 ± 5% of HL-60 cells stained positively for FITC-labeled annexin V with 0.2 μM TAS-103. With 1 μM, almost all cells were annexin positive, but about 30% of them were also PI positive. Morphological examination of FACS-analyzed samples with fluorescence microscopy showed annexin V staining localized exclusively to the cell membrane, with no staining in the cytoplasm (data not shown). The PS flip-flop may be connected with the activation of caspase-3, as shown above. Treatment of the wells with 25 nM TAS-103 and caspase-3 inhibitor DEVD-fmk reduces the number of annexin-positive cells by 18 ± 7%. The appearance of outer leaflet PSs apparently requires caspase activation (36, 37).

DNA Fragmentation. The DNA of HL-60 cells treated with TAS-103 was extracted and analyzed by electrophoresis on agarose gels. The gel presented in Fig. 9 shows unambiguously that the genomic DNA of the drug-treated cells was severely fragmented, even when using a drug concentration as low as 25 nM. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis (38). We observed similar patterns of DNA internucleosomal fragmentation with camptothecin and etoposide (22). Another detection system, the TUNEL assay, was also used to characterize DNA strand breaks in situ. The TUNEL method is based on the fluorescein labeling of apoptotic DNA fragments (39, 40). This assay is capable of detecting DNA strand breaks that

50% cleavage, respectively (data not shown). The cleavage of PARP occurs essentially on treatment of the cells with concentrations of ≥25 nM TAS-103, i.e., the concentrations for which the hypodiploid peak (sub-G₁) starts to appear in the cell cycle experiments. There is little or no cleavage of PARP with low drug concentrations (range, 2–20 nM), which cause a profound G₂ arrest. Therefore, we are inclined to believe that the cleavage of PARP might be a sign that the cell is unable to cope with a saturating DNA injury arising from the topoisomerase-directed genotoxic insult. With drug concentrations of ≥25 nM, the damages caused to the genetic material in the HL-60 cells are too frequent/abundant to be repaired, and therefore the cells engage in the irreversible apoptotic pathway.

In parallel, we followed by immunoblot analysis the proteolytic activation of pro-caspase-3 (Fig. 7b) and the intracellular levels of caspase-8 (Fig. 7c) in HL-60 cells treated with TAS-103. A distal caspase such as caspase-3 can be directly activated by a proximal caspase such as caspase-8 (29, 30). Caspase-8 is synthesized as two isoforms of Mr ~55,000. In both cases, a concentration of 50 nM TAS-103 was sufficient to induce quantitative cleavage of pro-caspase-3 and caspase-8 after a 24-h incubation period. These experiments indicate that TAS-103 activates both distal and proximal caspasess. The activation of caspase-8 shown by the Western blot experiments in Fig. 7c was independently confirmed by colorimetry using the N-acetyl-Ile-Glu-Thr-Asp-pNA peptide substrate (data not shown).

Reduced Expression of bcl-2. The bcl-2 oncoprotein located on the outer mitochondrial membrane is important for the suppression of apoptosis and mitochondrial manifestations of apoptosis (31). bcl-2 prevents the initiation of the cellular apoptotic program by stabilizing the mitochondrial permeability transition and avoiding the subsequent release of cytochrome c to prevent caspase activation (32). In doing so, the protein can block apoptotic death in multiple contexts, including the case of topoisomerase II inhibitors (33). The immunoblot analysis presented in Fig. 7d indicates that treatment of the cells with TAS-103 induces a down-regulation of bcl-2. Low drug concentrations (25 nM) suffice to considerably reduce the level of bcl-2 proteins in HL-60 cells. This effect must facilitate caspase activation.

Externalization of PSs. PS lipids are normally confined to the inner leaflet of the plasma membrane but are exported to the outer plasma membrane leaflet during apoptosis to serve as a trigger for recognition of apoptotic cells by phagocytes (34). PS can be detected by staining with a FITC conjugate of annexin V, a Mr 38,000 protein that binds naturally to PS (35). HL-60 cells treated with TAS-103 for 24 h were found to be positive for PS in the outer leaflet (Fig. 8). Approximately 55 ± 5% of HL-60 cells stained positively for FITC-labeled annexin V with 0.2 μM TAS-103. With 1 μM, almost all cells were annexin positive, but about 30% of them were also PI positive. Morphological examination of FACS-analyzed samples with fluorescence microscopy showed annexin V staining localized exclusively to the cell membrane, with no staining in the cytoplasm (data not shown). The PS flip-flop may be connected with the activation of caspase-3, as shown above. Treatment of the wells with 25 nM TAS-103 and caspase-3 inhibitor DEVD-fmk reduces the number of annexin-positive cells by 18 ± 7%. The appearance of outer leaflet PSs apparently requires caspase activation (36, 37).

DNA Fragmentation. The DNA of HL-60 cells treated with TAS-103 was extracted and analyzed by electrophoresis on agarose gels. The gel presented in Fig. 9 shows unambiguously that the genomic DNA of the drug-treated cells was severely fragmented, even when using a drug concentration as low as 25 nM. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis (38). We observed similar patterns of DNA internucleosomal fragmentation with camptothecin and etoposide (22). Another detection system, the TUNEL assay, was also used to characterize DNA strand breaks in situ. The TUNEL method is based on the fluorescein labeling of apoptotic DNA fragments (39, 40). This assay is capable of detecting DNA strand breaks that...
occur before nucleus fragmenting. Control cells containing intact genomic DNA appeared red (due to the staining with PI), whereas about 30% apoptotic cells colored green were detected by flow cytometry within the population of cells treated with 25 nM TAS-103 for 24 h (Fig. 10). At this concentration, the incorporation of FITC-dUTP was detected mainly in S phase. In addition, the bivariate analysis of DNA strand breaks (FITC) and DNA content (PI) by flow cytometry reveals that treatment with lower concentrations of TAS-103 (2–10 nM) induces an accumulation of cells in the G2-M phase but does not produce TUNEL-positive cells. An increased DNA fragmentation, as judged from the TUNEL assay coupled with a reciprocal decrease in ΔΨmt and the G2-M cell population, suggests that as the drug concentration increases, there is an inappropriate exit from an arrested G2 stage to the next stage that causes apoptotic cell death in these HL-60 cells. DNA fragmentation is detected only on treatment with drug concentrations that cause apoptotic cell death in these HL-60 cells. DNA fragmentation is induced by TAS-103 (2–10 nM) and not by drug concentrations that cause G2 arrest, whereas higher concentrations result in apoptosis. It is quite common for an anticancer drug to affect both cell cycle and apoptotic machinery.

We demonstrate that the anticancer drug TAS-103 is a potent inducer of apoptosis in HL-60 human leukemia cells. The induction of apoptosis is associated with (a) loss of the mitochondrial potential membrane (ΔΨmt), (b) decrease in intracellular pH, (c) down-regulation of bcl-2, and (d) activation of caspase-3 and -8. It is likely that several other cysteine proteases are implicated as well. The family of intracellular signaling molecules whose activity is regulated during apoptosis is increasing rapidly and includes, for example, a variety of protein kinases (41). It is not reasonable to analyze all these factors, but it is good to check at least a few of them to try to establish correlations. Here we have focused our efforts on ΔΨmt, intracellular pH, and caspase-3, which may be connected. In normal circumstances, most of the mitochondrial pores are closed. The fluorescence measurements with the probes DiOC6, Mitotracker Red, and JC-1 reveal that TAS-103 causes the pores to open, and this has dramatic consequences on mitochondria physiology. The collapse of ΔΨmt results in an uncoupling of the respiratory chain and the efflux of small molecules (e.g., cytochrome c and calcium) and certain proteins including caspase-2 and -9 (42) as well as the apoptosis-inducing factor that can, in turn, stimulate the proteolytic activation of caspase-3 (43). Protons will also escape from the mitochondria and will be released in the cytosol, contributing to the intracellular acidification process that we monitored by fluorescence using the SNARF probe. Lowering the pH can induce apoptosis in HL-60 cells (44). Intracellular acidification is often considered to be a consequence of the mitochondrial proton leak. However, it is possible that drug-induced acidification is a cause rather than a consequence of the loss of ΔΨmt (45). Indeed, a drug-induced increase in the proton permeability of the mitochondrial inner membrane could lead to a decrease in the mitochondrial membrane potential (46). Nevertheless, we can plausibly envisage that the pH serves to modulate the apoptotic responsiveness of the cell as well as amplify the apoptotic program.

An interesting link may be established between the cell cycle experiments and the ΔΨmt measurements. The results in Fig. 3 indicate that low concentrations (2–20 nM) of TAS-103 induce G2 cell cycle arrest, whereas higher concentrations (>30 nM) lead to apoptosis, as judged from the appearance of the hypodiploid DNA content peak. In parallel, we observe that low concentrations of TAS-103 provoke an increase in ΔΨmt, whereas higher concentrations result in a marked decrease of ΔΨmt. The similarity between the two set of data strongly suggests that the two events are correlated. To our knowledge, such a correlation between G2 arrest and ΔΨmt has never been reported previously.

REFERENCES


DISCUSSION

TAS-103 is a potent inhibitor of mammalian topoisomerases, acting preferentially against topoisomerase II. Recent studies have shown that the drug was equipotent to etoposide against topoisomerase IIα (6). At the cellular level, the inhibition of this enzyme produces multiple DNA strand breaks that may represent apoptosis-activating signals. Here we show that these signals involve changes in the cell cycle components. TAS-103 induces G2 cell cycle arrest before cell death. It is quite common for an anticancer drug to affect both cell cycle and apoptotic machinery.

We demonstrate that the anticancer drug TAS-103 is a potent inducer of apoptosis in HL-60 human leukemia cells. The induction of apoptosis is associated with (a) loss of the mitochondrial potential membrane (ΔΨmt), (b) decrease in intracellular pH, (c) down-regulation of bcl-2, and (d) activation of caspase-3 and -8. It is likely that several other cysteine proteases are implicated as well. The family of intracellular signaling molecules whose activity is regulated during apoptosis is increasing rapidly and includes, for example, a variety of protein kinases (41). It is not reasonable to analyze all these factors, but it is good to check at least a few of them to try to establish correlations. Here we have focused our efforts on ΔΨmt, intracellular pH, and caspase-3, which may be connected. In normal circumstances, most of the mitochondrial pores are closed. The fluorescence measurements with the probes DiOC6, Mitotracker Red, and JC-1 reveal that TAS-103 causes the pores to open, and this has dramatic consequences on mitochondria physiology. The collapse of ΔΨmt results in an uncoupling of the respiratory chain and the efflux of small molecules (e.g., cytochrome c and calcium) and certain proteins including caspase-2 and -9 (42) as well as the apoptosis-inducing factor that can, in turn, stimulate the proteolytic activation of caspase-3 (43). Protons will also escape from the mitochondria and will be released in the cytosol, contributing to the intracellular acidification process that we monitored by fluorescence using the SNARF probe. Lowering the pH can induce apoptosis in HL-60 cells (44). Intracellular acidification is often considered to be a consequence of the mitochondrial proton leak. However, it is possible that drug-induced acidification is a cause rather than a consequence of the loss of ΔΨmt (45). Indeed, a drug-induced increase in the proton permeability of the mitochondrial inner membrane could lead to a decrease in the mitochondrial membrane potential (46). Nevertheless, we can plausibly envisage that the pH serves to modulate the apoptotic responsiveness of the cell as well as amplify the apoptotic program.

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Fig. 10. Effects of TAS-103 on nuclear DNA fragmentation. Cells were stained with PI and assessed for nuclear DNA cleavage on a per-cell basis using the TUNEL technique.
Apoptotic Response of HL-60 Human Leukemia Cells to the Antitumor Drug TAS-103

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