Selective Induction of Apoptosis in Hep 3B Cells by Topoisomerase I Inhibitors: Evidence for a Protease-dependent Pathway that Does Not Activate Cysteine Protease P32

Philip N. Adjei,* Scott H. Kaufmann,‡ Wai-Yee Leung,§ Fei Mao,§ and Gregory J. Gores*

**Division of Gastroenterology and Internal Medicine and ‡Division of Oncology Research, Mayo Clinic and Foundation, Rochester, Minnesota 55905; and §Molecular Probes, Inc., Eugene, Oregon 97402*

Abstract

Progress in the treatment of hepatocellular carcinoma (HCC), a common tumor worldwide, has been disappointing. Inhibitors of topoisomerases are being widely studied as potential inducers of tumor cell apoptosis. Our aims were to determine whether topoisomerase-directed drugs would induce apoptosis in a human HCC cell line (Hep 3B) and, if so, to investigate the mechanism. The topoisomerase I poison camptothecin (CPT) induced apoptosis of Hep 3B cells in a time- and concentration-dependent manner. In contrast, the topoisomerase II poison etoposide failed to induce apoptosis despite the apparent stabilization of topoisomerase II–DNA complexes. Unexpectedly, CPT-induced apoptosis in this cell type occurred without any detectable cleavage of poly(ADP-ribose) polymerase or lamin B, polypeptides that are commonly cleaved in other cell types undergoing apoptosis. Likewise, Hep 3B cell apoptosis occurred without a detectable increase in interleukin-1β–converting enzyme (ICE)-like or cysteine protease P32 (CPP32)-like protease activity. In contrast, trypsin-like protease activity (cleavage of Boc-Val-Leu-Lys-chloromethylaminocoumarin in situ) increased threefold in cells treated with CPT but not etoposide. Tosyl-lysyl chloromethyl ketone inhibited the trypsin-like protease activity and diminished CPT-induced apoptosis. These data demonstrate that *(a)* apoptosis is induced in Hep 3B cells after stabilization of topoisomerase I-DNA complexes but not after stabilization of topoisomerase II-DNA complexes as measured by alkaline filter elution; *(b)* Hep 3B cell apoptosis occurs without activation of ICE-like and CPP32-like protease activity; and (c) a trypsin-like protease activity appears to contribute to apoptosis in this cell type. *(J. Clin. Invest.* 1996. 98:2588–2596.) Key words: camptothecin • CPP32 • etoposide • interleukin-1β–converting enzyme • poly(ADP-ribose) polymerase

Introduction

Hepatocellular cancer (HCC),[1] a neoplasm which usually occurs in the setting of viral liver disease with cirrhosis, is one of the most common cancers worldwide (1). Many patients with HCC present with metastatic disease and are no longer candidates for curative surgical resection (2). Furthermore, even in those patients with HCC localized to the liver, surgical resection is often not feasible due to the severity of the underlying parenchymal liver disease (2). A variety of therapeutic modalities for unresectable HCC have been tested, including numerous systemic chemotherapeutic agents, hepatic artery chemoembolization, radiation therapy, and lymphokine-activated killer cells, all without significant success (3–6). Survival rates worldwide are a year or less for patients with advanced disease, indicating the lack of impact of current therapies on survival.

Recent data suggest that a wide variety of chemotherapeutic agents exert their effects by inducing apoptosis (7–12). Information regarding the cellular, biochemical, and molecular mechanisms of apoptosis in HCC is scant. It is known that p53 mutations are frequent in HCC (13–16). Mutations of p53 block the induction of apoptosis by chemotherapeutic agents in many cell types (17) and may contribute to the resistance of hepatocellular cancers to current chemotherapeutic agents.

Topoisomerase poisons, particularly topoisomerase I inhibitors, induce apoptosis in many cells that lack functional p53 (18–20). Topoisomerases are ubiquitous enzymes that modulate the topographic structure of DNA by transiently introducing breaks in the DNA backbone (21, 22). Two classes of topoisomerases exist in eukaryotic cells: type I enzymes, which cause single-strand DNA breaks, and type II enzymes, which cause double-strand DNA breaks. Treatment of cells with agents termed "topoisomerase poisons" (23) results in the stabilization of covalent topoisomerase-DNA intermediates. In a manner that is currently not well understood, these covalent topoisomerase-DNA intermediates trigger the apoptotic machinery of the cell, resulting in cell death (18–20, 24). More recently, treatment with β-lapachone, which inhibits topoisomerase I but does not stabilize topoisomerase I-DNA covalent complexes, has also been reported to induce apoptosis in certain cell types in a p53-independent fashion (25, 26).

Apoptosis induced by topoisomerase-directed agents, like apoptosis induced by other drugs, is accompanied by intracel-

Address correspondence to Gregory J. Gores, Center for Basic Research in Digestive Diseases, Mayo Clinic and Foundation, Rochester, MN 55905. Phone: 507-284-0680; FAX: 507-284-0762; E-mail: gores.gregory@mayo.edu

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1. *Abbreviations used in this paper:* AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; CMAC, 7-amino-4-chloromethylcoumarin; CPP32, cysteine protease P32; HCC, hepatocellular cancer; ICE, IC-1β–converting enzyme; PARP, poly(ADP ribose) polymerase; RT-PCR, reverse transcription PCR; TLCK, tosyl-lysyl chloromethyl ketone.

lular protease activation (27–29). Recent attention has focused on members of the interleukin-1β converting enzyme (ICE) protease family as potential apoptotic proteases (30–33). To date, cDNAs for 10 human ICE family members have been cloned. Based on preferential catalytic activity, they can be divided into two broad groups, ICE-like family members, which preferentially recognize the YVAD tetrapeptide sequence, and CPP32-like family members, which preferentially recognize the DEVD tetrapeptide sequence.

In the present study we used human Hep 3B cells, which like many human hepatocellular cancers are hepatitis B surface antigen (HBsAg) positive and lack functional p53 (15, 34), to address the following questions: (*a*) do Hep 3B cells express both topoisomerase I and II enzymes, and do topoisomerase I and II inhibitors stabilize topoisomerase-DNA adducts in these cells? (*b*) Does stabilization of topoisomerase-DNA adducts result in apoptosis in Hep 3B cells? (*c*) Do protease inhibitors prevent apoptosis induced by topoisomerase inhibitors? (*d*) What are the specific proteases that are activated during treatment of Hep 3B cells with topoisomerase inhibitors?

Methods

Cells. Hep 3B cells were cultured in minimum essential medium containing 10% fetal bovine serum and 100 nM insulin in 5% CO_2-95% air at 37°C. For morphological studies, cells were cultured as previously described (35). The human leukemia cell lines HL-60 and THP-1 (American Type Culture Collection, Rockville, MD) were grown as suspension cultures in RPMI-1640 containing 10% fetal bovine serum without (HL-60) or with (THP-1) 50 μM β-mercaptoethanol.

Solutions. Nucleic acid isolation buffer consisted of 50 mM Tris-HCl, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecylsulfate and 1 mg/ml proteinase K, pH 9. Alkylation buffer consisted of 6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C), 10 mM EDTA, 1 mM PMSF and 1% (vol/vol) β-mercaptoethanol (36). Hypotonic buffer consisted of 25 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.5.

Morphologic and biochemical assessment of apoptosis. Nuclear changes indicative of apoptosis were quantitated using fluorescent microscopy and the nuclear fluorescent binding dye 4,6-diamidino-2-phenylindole (DAPI) as we have previously described in detail (37–39). Results were confirmed by confocal microscopy. DNA was isolated from cultured cells and separated by agarose gel electrophoresis as we have also previously described in detail (18, 38).

Measurement of ICE-like and cysteine protease P32 (CPP32)-like protease activity in cytosol. After treatment with drug or diluent, cells were scraped off the plates into drug-free medium using a cell lifter. The contents from five 100-mm dishes were combined. All subsequent steps were performed at 4°C. Cells were pelleted at 200 *g* for 10 min, washed twice with 5 ml of serum-free medium, and resuspended in 1.25 ml of hypotonic buffer containing freshly added 0.5 mM PMSF, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. After a 20-min incubation, cells were lysed by 100 strokes using a 2-ml Dounce homogenizer with a tight-fitting pestle. Once cell lysis was confirmed by phase contrast microscopy, the homogenate was centrifuged at 3,000 *g* for 3 min to remove nuclei, then at 227,000 *g* for 1 h in an ultracentrifuge (TL-100; Beckman Instruments, Palo Alto, CA) to remove mitochondria, lysosomes, and microsomes. Dithiothreitol was added to the supernatant to a final concentration of 2 mM, and samples were frozen at −70°C. Protease activity was measured by adding 50 μg of cytosolic protein to 1.5 ml of buffer containing 25 mM Hepes (pH 7.5), 10 mM dithiothreitol, 0.1% CHAPS, 0.5 mM PMSF, 100 U/ml Trasylol, and 20 μM Ac-Tyr-Val-Ala-Asp-AMC (AMC = 7-amino-4-methylcoumarin) (to measure ICE-like protease activity) or 20 μM Z-Asp-Glu-Val-Asp-AFC (AFC, 7-amino-4-trifluoromethylcoumarin) (to measure CPP32-like protease activity). Fluorescence was monitored using a fluorometer (450; Sequoia-Turner, Mountain View, CA) with excitation and emission wavelengths of 360 and 480 nm for AMC and 400 and 505 nm for AFC. Standard curves were generated with AMC and AFC (40). Cytosolic extracts from the THP-1 human monocytic leukemia cell line and from etoposide-treated HL-60 cells were used as positive controls for ICE-like and CPP32-like protease activity, respectively (41–43).

Measurement of intracellular trypsin-like protease activity. Intracellular single-cell trypsin-like protease activity was measured using the fluorogenic protease substrate Z-Val-Leu-Lys-CMAC (CMAC, 7-amino-4-chloromethylcoumarin) and multiparameter digitized video microscopy analogous to the technique previously developed and validated by us for measuring intracellular calpain activity (44). Intracellular trypsin-like protease activity was measured by adding 50 μM Z-Val-Leu-Lys-CMAC to the media and quantitating intracellular fluorescence over 15 min using digitized video microscopy and excitation and emission wavelengths of 380 and 460 nm, respectively.

Z-Val-Leu-Lys-CMAC was synthesized in five steps as described below. All steps were performed at room temperature unless indicated. Melting point measured on a Mel-Temp (Laboratory Devices, Holliston, MA) was uncorrected. ^1H nuclear magnetic resonance (NMR) spectrum was recorded on a 400-MHz Bruker (Billerica, MA) FT NMR spectrometer. HPLC analysis was performed on a Waters (Milford, MA) system using a Hamilton reverse phase PRP-1 column (4.1 × 250 mm) eluted at a rate of 1 ml/min with MeCN:0.1% TFA (5:95 to 95:5) with UV monitoring at 254 and 329 nm. (*Step 1*) Synthesis of Z-Val-Leu-*O*-Succinimide: A mixture of Z-Val-Leu-OH (13.7 mmol), *N*-hydroxysuccinimide (15.1 mmol) and 1,3-dicyclohexylcarbodiimide (16.6 mmol) in EtOAc (200 ml) was stirred overnight. The resulting suspension was suction filtered; and the filtrate was vacuum evaporated to dryness to give Z-Val-Leu-*O*-Succinimide as a colorless solid (13.7 mmol, 99% yield). (*Step 2*) Synthesis of Z-Val-Leu-Lys(Boc)-OMe: A mixture of Z-Val-Leu-*O*-Succinimide (13.7 mmol), H-Lys(Boc)-OMe·HCl (15.1 mmol) and triethylamine (16.6 mmol) in $CHCl_3$ (300 ml) was stirred for 1.5 h. The solvent was removed in vacuo; and the residue was purified by column chromatography on silica gel using 2% MeOH in $CHCl_3$ as eluent to give Z-Val-Leu-Lys(Boc)-OMe as a colorless solid (14.1 mmol, 98%). (*Step 3*) Synthesis of Z-Val-Leu-Lys(Boc)-OH: To a suspension of Z-Val-Leu-Lys(Boc)-OMe (14.1 mmol) in MeOH (500 ml) was added a solution of cesium carbonate (28.1 mmol) in H_2O (250 ml). After the mixture was stirred for 3 h, the methanol was removed by vacuum evaporation and the residue was diluted with 1 liter H_2O. The aqueous layer was acidified to pH 2 with 10% HCl and extracted with EtOAc (3 × 200 ml). The combined EtOAc layers were washed with H_2O (100 ml) and saturated NaCl (100 ml), then dried with anhydrous Na_2SO_4. Removal of the solvent in vacuo afforded Z-Val-Leu-Lys(Boc)-OH as a colorless solid (12.5 mmol, 89%). (*Step 4*) Synthesis of Z-Val-Leu-Lys(Boc)-CMAC: To a solution of Z-Val-Leu-Lys(Boc)-OH (6.76 mmol) in DMF/pyridine (1:1, 60 ml) at 0°C was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (7.43 mmol). The mixture was stirred at 0°C for 15 min and then CMAC (3.38 mmol) was added. The solution was stirred at 0°C for 3 h, then poured into a mixture of 5% HCl (500 ml) and EtOAc (200 ml). The aqueous layer was extracted with EtOAc (2 × 150 ml). The combined organic layers were washed with 5% HCl (2 × 100 ml), H_2O (5 × 100 ml) and saturated NaCl (100 ml), dried with anhydrous Na_2SO_4, filtered and evaporated to dryness in vacuo. The residue was purified by column chromatography on silica gel eluted with 5% MeOH in $CHCl_3$ to give Z-Val-Leu-Lys(Boc)-CMAC as a colorless viscous oil (0.38 mmol, 12%). (*Step 5*) Synthesis of Z-Val-Leu-Lys-CMAC·HCl; To Z-Val-Leu-Lys(Boc)-CMAC (0.38 mmol) in a 50-ml round bottom flask at 0°C was added trifluoroacetic acid (5 ml). After 30 min, the solvent was removed in vacuo. The residue was dissolved in MeOH (3 ml) with concentrated HCl (0.1 ml) and then added dropwise to vigorously stirred Et_2O (60 ml). The product, Z-Val-Leu-

Lys-CMAC·HCl, was collected by centrifugation as off-white solid (0.27 mmol, 70%); mp 193–195°C; HPLC purity 98%; ^{1}H NMR (DMSO-d_6 with D_2O exchange) d 8.40–7.75 (m, 8H), 7.66–7.55 (m, 1H), 7.35–7.20 (m, 5H), 6.55 (s, 1H), 5.10–4.90 (m, 4H), 4.40–4.28 (m, 2H), 3.90–3.80 (m, 1H), 2.80–2.65 (m, 2H), 2.00–1.15 (m, 10H), 0.95–0.70 (m, 12H).

Alkaline elution. Logarithmically growing Hep 3B cells labeled for 24 h with 1 μM [^{14}C]thymidine in minimum essential medium were trypsinized, sedimented, and incubated for 2 h at 37°C in fresh medium. Aliquots were then incubated at 37°C for 30 min with the indicated concentration of camptothecin or etoposide, then deposited by gentle suction on Nucleopore phosphocellulose filters (1 μm pore size). Cells were lysed by allowing 5 ml of buffer consisting of 1% (wt/vol) SDS, 100 mM glycine, 25 mM EDTA (pH 10), and 0.5 mg/ml proteinase K to drip through the filters. After filters were washed with 20 mM EDTA (pH 10), DNA was eluted with 20 mM EDTA (adjusted to pH 12.1 with tetrapropylammonium hydroxide). Fractions consisting of eluate, the filter, and the tubing were analyzed as described (45). Cells that received 150–900 cGy of γ-irradiation at 4°C from a ^{137}Cs source were included in each experiment as a standard curve. For each drug treatment, the fraction of DNA remaining bound to the filter after the 7-h elution period was compared to this standard curve. In some experiments, duplicate samples were treated with lysis buffer in the absence and presence of proteinase K to determine whether the drug-induced strand breaks were protein-associated.

Immunoblotting. Cells cultured in 100-mm dishes were washed twice with serum-free medium and lysed in 3 ml of alkylation buffer. Samples were sonicated and prepared for electrophoresis as previously described (36). After separation on 5–15% (wt/vol) polyacrylamide gels in the presence of SDS, samples were transferred to nitrocellulose. Blots were probed as previously described (18) using mouse monoclonal antibodies to topoisomerase I (46), topoisomerase II (47), poly(ADP-ribose) polymerase (PARP) (48), CPP32 and Ich-1 (Transduction Laboratories, Lexington, KY), rabbit antibodies against Mch-3 (49) and ICE (Oncogene Science, Boston, MA) as well as chicken polyclonal antibodies to lamin B (50).

Reverse transcription (RT)-PCR. Primers (5′ and 3′, respectively) used for PCR included ICE nucleotides (nts.) 11–28 and 396–413, ICE-rel$_{II}$ nts. 32–49 and 419–436, Ich-1$_L$ nts. 13–30 and 419–436, CPP32β nts. 56–73 and 487–504, Mch2α nts. 66–83 and 502–519, and Mch3α nts. 30–46 and 434–451 (51–56). These primers were designed to encompass the prodomain and the amino-terminal half of the respective P20 subunits, regions of the cDNAs that display relatively low sequence homology between various ICE family members. Total RNA was isolated using the guanidinium isothiocyanate method (57). cDNA was synthesized using reverse transcriptase (Life Sciences, Gaithersburg, MD) and 10 μg of total RNA as suggested by the supplier. cDNAs were amplified using AmpliTaq polymerase from Perkin-Elmer (Applied Biosystems Division, Foster City, CA) with 35 cycles in a Peltier thermal cycler (PTC-200; MJ Research Inc., Watertown, MA). The integrity of the RNA was checked by PCR amplification of the housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase. 15 μl of the amplification products were analyzed on a 1% agarose gel and photographed using UV illumination.

Statistical analysis. All data are expressed as mean±SEM from at least three separate experiments. Differences between groups were analyzed using Student's *t* test when only two groups were analyzed or an analysis of variance for repeated measures if three or more groups were analyzed. When analysis of variance was performed, a post hoc Bonferroni test was used to correct for multiple comparisons. All statistical analyses were performed using Instat Software (GraphPAD, San Diego, CA).

Materials. Chemicals of analytical grade purity were purchased from the following suppliers: Zinc sulfate, Tris-HCl, EDTA, ribonuclease A, leupeptin, *N*-tosyl-L-phenylalanine chloromethyl ketone, tosyl-lysyl chloromethyl lactone (TLCK), E-64, pepstatin, camptothecin, etoposide, 1,10-phenanthroline, and ethidium bromide from Sigma Chemical Co. (St. Louis, MO); 3,4-dichloroisocoumarin, PMSF, 4-(amidinophenyl) methanesulfonyl fluoride, proteinase K, and collagenase type D from Boehringer Mannheim (Indianapolis, IN); CMAC was from Molecular Probes (Eugene, OR); cesium carbonate from Aldrich (Milwaukee, WI); *N*-hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and 1,3-dicyclohexylcarbodiimide from Advanced ChemTech Corp. (Louisville, KY);

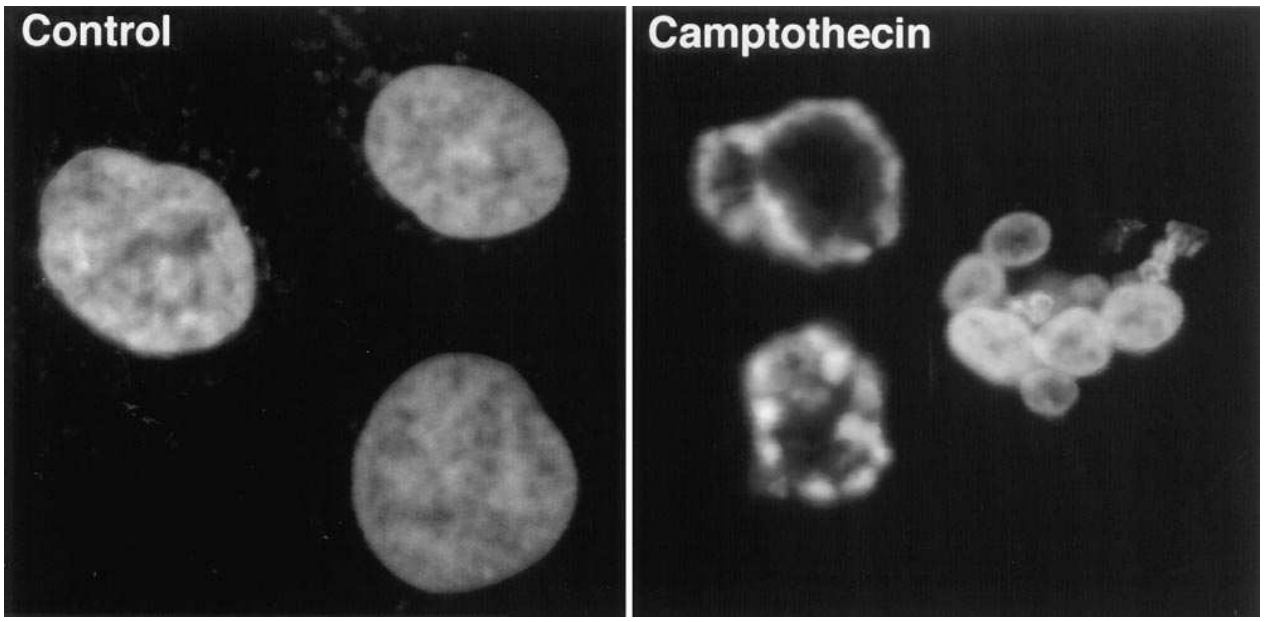

Figure 1. Confocal microscopy of Hep 3B cells. Hep 3B cells were cultured in medium without (*left*) or with (*right*) 1 μM camptothecin for 48 h at 37°C. Cells were stained with DAPI and viewed by confocal microscopy. Note the spherical nuclei and uniform chromatin distribution within the nuclei of control cells (*left*). In contrast, camptothecin-treated cells demonstrate chromatin condensation, margination, and nuclear fragmentation (*right*).

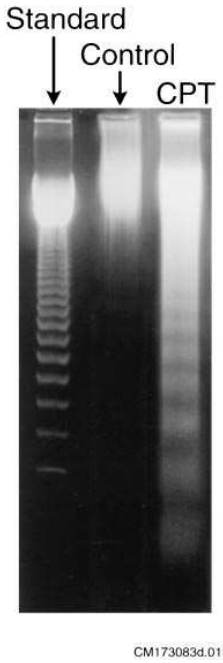

Figure 2. Internucleosomal DNA cleavage in camptothecin-treated Hep 3B cells. DNA was extracted from cells incubated in the presence and absence of 1 μM camptothecin (CPT) for 48 h at 37°C. The ladder pattern of DNA fragmentation was observed in camptothecin-treated cells (*right lane*) but not in controls (*middle lane*). The left lane contains molecular weight markers.

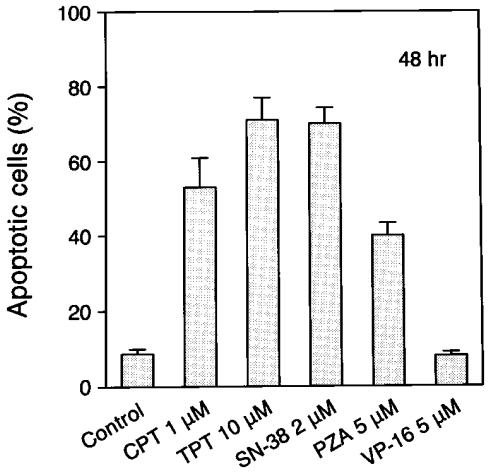

Figure 4. Induction of apoptosis in Hep 3B cells by topoisomerase inhibitors. Hep 3B cells were incubated for 48 h with the topoisomerase I inhibitors camptothecin (*1 μM*), topotecan (*10 μM*), or SN-38 (*2 μM*); the topoisomerase II inhibitor etoposide (*5 μM*); and the dual topoisomerase inhibitor pyrazoloacridine (*5 μM*). After 48 h, apoptosis was quantified as described in Methods.

Z-Asp-Glu-Val-Asp-AFC, and Z-Asp-Glu-Val-Asp-fmk from Enzyme Systems Products (Dublin, California); and Ac-Tyr-Val-Ala-Asp-AMC, Ac-Tyr-Val-Ala-Asp-cmk, Z-Val-Leu-OH, and H-Lys (Boc)-OMe from Bachem Bioscience (King of Prussia, PA). Topotecan (TPT) and SN-38 were kindly provided by SmithKline Beecham (King of Prussia, PA) and Pharmacia/Upjohn (Kalamazoo, MI), respectively. Pyrazoloacridine (PZA) was a gift from Dr. Judith Sebolt-Leopold (Warner-Lambert, Plains, NJ).

Results

Do topoisomerase-directed agents induce apoptosis of Hep 3B cells? After treatment of Hep 3B cells with 1 μM camptothecin for 48 h, confocal microscopy revealed the classical nuclear morphological features of apoptosis including chromatin margination/condensation and nuclear fragmentation (Fig. 1). Internucleosomal DNA fragmentation was also observed in camptothecin-treated cells (Fig. 2). Further experiments revealed that induction of apoptosis by camptothecin was time and concentration dependent (Fig. 3). Maximal apoptosis was observed at 48 h with 1 μM camptothecin, with 53±8% of cells appearing apoptotic. Therefore, we used this concentration and time point for the remainder of the studies. Apoptosis of Hep 3B cells was also observed with the camptothecin analogs topotecan and SN-38 as well as the dual topoisomerase I and II inhibitor pyrazoloacridine (58), but not with the topoisomerase II poison etoposide (Fig. 4). These results suggest that induction of apoptosis in Hep 3B cells is selective for topoisomerase I-directed agents.

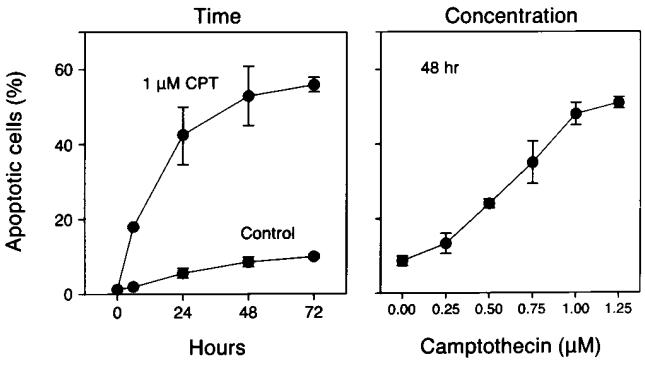

Figure 3. Time course and concentration dependence of camptothecin-induced apoptosis in Hep 3B cells. Hep 3B cells were incubated in the presence or absence of camptothecin. Cells undergoing apoptosis were quantified as described in Methods. Induction of apoptosis by 1 μM CPT was time-dependent, with maximum apoptosis observed at 48 h (*left*). Induction of apoptosis by CPT was concentration dependent, with a maximal effect at 1 μM CPT (*right*).

Do Hep 3B cells express both topoisomerase I and II enzymes; and do topoisomerase II poisons stabilize topoisomerase II-DNA complexes in these cells? To determine whether resistance of Hep 3B cells to induction of apoptosis by etoposide might be due to altered levels of topoisomerase II or diminished stabilization of covalent topoisomerase II-DNA complexes, these parameters were evaluated. Immunoblot analysis demonstrated that Hep 3B cells express readily detectable levels of both topoisomerase I and II (Fig. 5, *A* and *B*, lanes *1–3*). Consistent with previous results (59), we observed decreased levels of topoisomerase I and increased levels of topoisomerase II after treatment with camptothecin (Fig. 5, *A* and *B*, lanes *4–6*). In contrast, levels of topoisomerase I and II remained constant during treatment with etoposide (Fig. 5, *A* and *B*, lanes *7–9*).

Alkaline elution was used to search for the formation of strand breaks that accompanies drug-induced stabilization of topoisomerase-DNA complexes (reviewed in reference 60). Treatment with 1 μM camptothecin and 5 μM etoposide for 30 min resulted in similar levels of DNA single-strand breaks, 470±145 vs 516±143 cGy equivalents, respectively. Consistent with previous experiments using this technique to examine topoisomerase-mediated strand breaks (61, 62), omission of proteinase K from the lysis buffer resulted in a 50% decrease in the number of camptothecin-induced single-strand breaks detected and a 75% decrease in the number of etoposide-induced

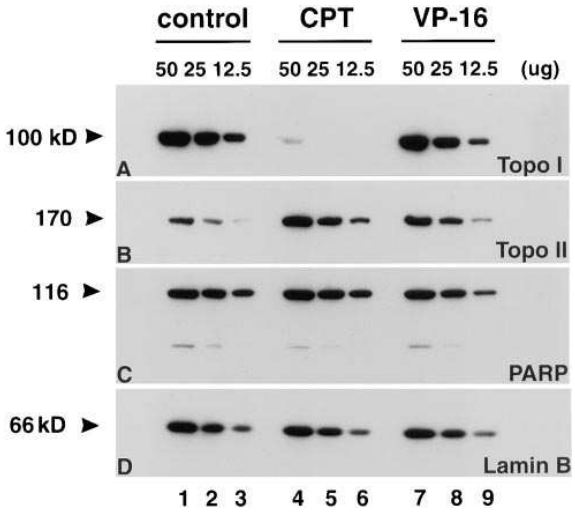

Figure 5. Immunoblot assay for cellular proteins from Hep 3B cells. Hep 3B cells were incubated for 48 h in the presence of 1 μM camptothecin, 5 μM etoposide, or diluent. Whole cell lysates were applied to adjacent wells on an SDS polyacrylamide gel. Samples contained 50 μg (lanes *1*, *4*, *7*), 25 μg (lanes *2*, *5*, *8*), or 12.5 μg (lanes *3*, *6*, *9*) of protein from cells treated with diluent (lanes *1–3*), camptothecin (lanes *4–6*), or etoposide (lanes *7–9*). Immunoblotting was performed with antibodies that recognize topoisomerase I (*A*), topoisomerase II (*B*), PARP (*C*), and lamin B (*D*).

single-strand breaks detected (data not shown), suggesting that a large portion of the nicked DNA in Hep 3B cells is covalently linked to protein. Collectively, these results are consistent with the view that both drugs enter Hep 3B cells and stabilize complexes between DNA and their respective enzymes. Thus, resistance of Hep 3B cells to etoposide-induced apoptosis does not seem to reflect low topoisomerase II levels or inability of etoposide to stabilize topoisomerase-DNA complexes in these cells. Instead, it appears to reflect a selective failure to activate apoptotic pathways following topoisomerase II-DNA complex stabilization.

Table I. Effect of Protease Inhibitors on Induction of Apoptosis in Camptothecin-treated Hep 3B Cells

Protease inhibitor	Concentration	Apoptotic cells, percentage of camptothecin-treated cells
Leupeptin	100 μM	98±2
Pepstatin	100 μM	94±1
TLCK	100 μM	43±3
TPCK	10 μM	Toxic
DCI	10 μM	Toxic
PMSF	1 mM	107±2
E-64	100 μM	104±2
CBZ-Leu-Leu-Tyr-CHN2	10 μM	104±1
1,10-Phenanthroline	1 mM	92±10
Z-Asp-Glu-Val-Asp-fmk	100 μM	99±1
Ac-Tyr-Val-Ala-Asp-cmk	75 μM	80±2
Ac-Tyr-Val-Ala-Asp-cmk plus TLCK	75 μM 100 μM	44±5

Values are means±SE. Hep 3B cells were incubated for 48 h in the presence of 1 μM camptothecin plus the indicated protease inhibitor of interest. After staining with 5 μM DAPI, samples were viewed by fluorescence microscopy. The number of cells undergoing apoptosis in the presence of protease inhibitors was expressed as a percentage of cells undergoing apoptosis after camptothecin treatment in the absence of protease inhibitors (53±8%). *TPCK*, *N*-tosyl-L-phenylalanine chloromethyl ketone; *DCI*, 3,4-dichloroisocoumarin.

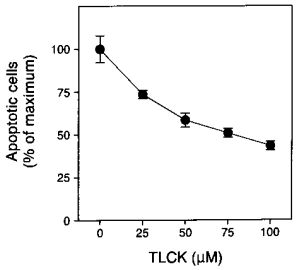

Figure 6. TLCK partially blocks camptothecin-induced apoptosis of Hep 3B cells. Hep 3B cells were incubated for 48 h with 1 μM camptothecin and the indicated concentrations of TLCK. Apoptosis was quantified as described in Methods. The number of cells undergoing apoptosis is expressed as a percentage of the number of cells undergoing apoptosis during exposure to 1 μM camptothecin in the absence of TLCK.

Do protease inhibitors prevent camptothecin-induced apoptosis in Hep 3B cells? Because proteases are thought to be important for the initiation and/or completion of apoptosis (reviewed in references 30–33, 63), we next evaluated the role of proteolysis in apoptosis of Hep 3B cells. In initial experiments, inhibitors of cysteine, serine, aspartate, and metalloproteases were evaluated for their ability to inhibit camptothecin-induced apoptosis in Hep 3B cells (Table I). Of all the inhibitors studied, only TLCK significantly inhibited apoptosis in camptothecin treated Hep 3B cells (Table I). Further experiments revealed that the effect was dependent on the TLCK concentration, with 100 μM TLCK diminishing the number of apoptotic cells in camptothecin-treated cultures by 57% (Fig. 6). In contrast, Ac-Tyr-Val-Ala-Asp-chloromethylketone (cmk), an inhibitor of ICE-like protease activity, only minimally inhibited camptothecin-induced apoptosis; and the combination of Ac-Tyr-Val-Ala-Asp-cmk plus TLCK was no more effective in inhibiting apoptosis than TLCK alone (Table I). Z-Asp-Glu-Val-Asp-fluoromethylketone (fmk), an inhibitor of CPP32-like protease activity, had no effect on camptothecin-induced apoptosis of Hep 3B cells (Table 1).

Which proteases are activated in camptothecin-treated Hep 3B cells? To further evaluate the role of proteases in the CPT-induced apoptosis, cleavage of endogenous substrates for apoptotic proteases was monitored. PARP is cleaved by active CPP32-like enzymes in a variety of cell types undergoing apoptosis (reviewed in references 30, 31). Lamins are cleaved by Mch2α in a number of cell types undergoing apoptosis (18, 64). Surprisingly, immunoblotting failed to demonstrate any apoptosis-associated cleavage of either PARP (Fig. 5 *C*) or lamin B (Fig. 5 *D*) in camptothecin-treated Hep 3B cells.

In additional experiments, cytosol fractions from control and CPT-treated Hep 3B cells were assayed for ICE-like and CPP32-like protease activity using exogenous fluorogenic substrates. No increase of either activity was detected in camptothecin-treated Hep 3B cells (Table II) even though ICE-like protease activity was readily detected in cytosol from the monocyte cell line THP-1 (41, 42), and CPP32-like protease activity was observed in cytosol from etoposide-treated HL-60 cells using these assays. Furthermore, no inhibition of CPP32-like protease activity in the cytosol from the etoposide-treated HL-60 cells was observed after the addition of cytosol ob-

Table II. Assessment of ICE-like and CPP32-like Protease Activity in Camptothecin-treated Hep 3B Cells

Condition	ICE-like activity	CPP32-like activity
	pmol AMC (AFC) × mg protein^{-1} × min^{-1}	
Hep 3B cells		
+ camptothecin	1.6±0.3	1.0±0.1
− camptothecin	2.9±0.9	1.0±0.4
Fold difference	0.6	1
P value	NS	NS
HL-60 cells		
+ etoposide	—	136±26
− etoposide	—	3.6±1.0
Fold difference	—	38
P value	—	< 0.01
THP-1 cells	20±2.3	—

Values are means±SE. Hep 3B cells were treated with 1 μM camptothecin or diluent for 48 h. Cytosol was prepared from homogenized cells as described in Methods. ICE-like and CPP32-like protease activities were determined using the fluorogenic substrates Ac-Tyr-Val-Ala-Asp-AMC and Z-Asp-Glu-Val-Asp-AFC, respectively. Changes in fluorescence were monitored over 10 min. The THP-1 human monocyte cell line, which is known to express ICE (42, 43) was used as a positive control for the ICE assay. Aliquots of the human leukemia cell line HL-60 treated with 68 μM etoposide for 6 h served as a positive control for the assay of CPP32-like activity.

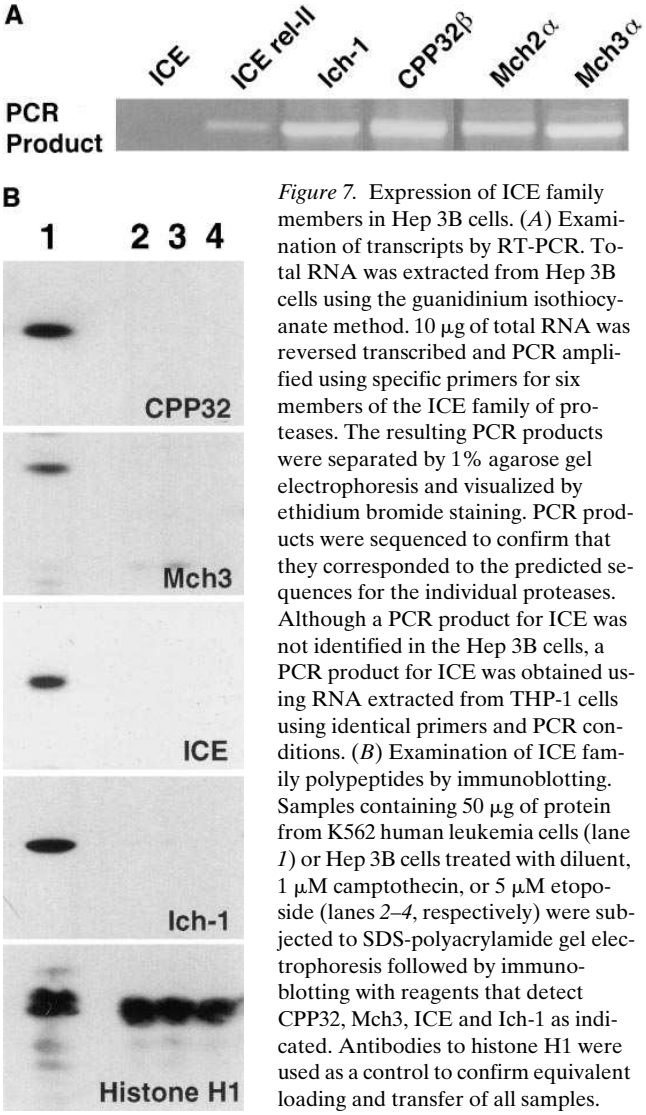

Figure 7. Expression of ICE family members in Hep 3B cells. (*A*) Examination of transcripts by RT-PCR. Total RNA was extracted from Hep 3B cells using the guanidinium isothiocyanate method. 10 μg of total RNA was reversed transcribed and PCR amplified using specific primers for six members of the ICE family of proteases. The resulting PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products were sequenced to confirm that they corresponded to the predicted sequences for the individual proteases. Although a PCR product for ICE was not identified in the Hep 3B cells, a PCR product for ICE was obtained using RNA extracted from THP-1 cells using identical primers and PCR conditions. (*B*) Examination of ICE family polypeptides by immunoblotting. Samples containing 50 μg of protein from K562 human leukemia cells (lane *1*) or Hep 3B cells treated with diluent, 1 μM camptothecin, or 5 μM etoposide (lanes *2–4*, respectively) were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with reagents that detect CPP32, Mch3, ICE and Ich-1 as indicated. Antibodies to histone H1 were used as a control to confirm equivalent loading and transfer of all samples.

tained from the Hep 3B cells, ruling out the possibility that Hep 3B cells contain an endogenous inhibitor of CPP32-like protease activity that interferes with the assay.

The lack of an increase in ICE-like and CPP32-like protease activity observed in apoptotic Hep 3B cells might reflect either a failure to express these proteases or a failure to activate them during CPT treatment. To distinguish between these two possibilities, expression of ICE family proteases was examined by RT-PCR and immunoblotting (Fig. 7). RT-PCR identified transcripts for ICErel-II, Ich-1, CPP32β, Mch2α, and Mch3α in Hep 3B RNA (Fig. 7 *A*). Sequencing of the RT-PCR products confirmed that they corresponded to mRNA for the indicated ICE-like proteases. In contrast, transcripts for ICE were not detected. Thus, ICE-like and CPP32-like protease transcripts are expressed by these cells. Western blotting, however, indicated that the levels of CPP32, Mch3, Ich-1, and ICE proenzymes were much lower in Hep 3B cells than in K562 human leukemia cells or any of a variety of human carcinoma cell lines examined (Fig. 7 *B* and data not shown).

Is a trypsin-like protease activity activated in camptothecin-treated Hep 3B cells? Because TLCK can inhibit trypsin-like proteases, and because previous results have suggested that trypsin-like protease activity might contribute to hepatocyte apoptosis induced by toxic bile salts (39), we measured intracellular trypsin-like protease activity in Hep 3B cells using Z-Val-Leu-Lys-CMAC and digitized video fluorescent microscopy. Trypsin-like protease activity increased threefold in camptothecin-treated cells (Fig. 8) but did not change from basal values in cells treated with etoposide ($P < 0.01$). Furthermore, this increase in intracellular trypsin-like protease activity was blocked by 100 μM TLCK (Fig. 8). These data suggest that a TLCK-inhibitable, trypsin-like protease is activated during camptothecin-induced apoptosis in Hep 3B cells. The failure of topoisomerase II-DNA adducts to lead to protease activation (Fig. 8) correlates with the inability of etoposide to induce apoptosis in these cells.

Discussion

It has been suggested that apoptosis occurs in four stages: (*a*) initiation by a cell death stimulus; (*b*) transduction of the stimulus through signaling pathways to activate the execution phase; (*c*) execution of the apoptotic process by effector proteases and endonucleases; and (*d*) a postmortem phase characterized by phagocytosis of the apoptotic bodies in vivo (65). In this study, we evaluated the initiation and effector phases of apoptosis in Hep 3B cells after treatment with topoisomerase poisons.

The initiation phase was evaluated by determining the ability of topoisomerase-directed agents to induce apoptosis. Unexpectedly, topoisomerase I–directed agents, but not topoisomerase II poisons, induced apoptosis in this cell line. The

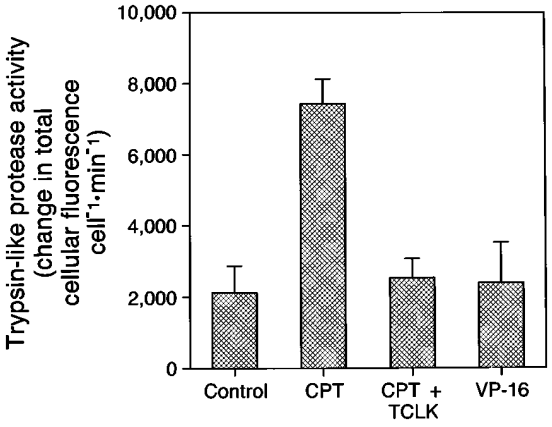

Figure 8. Trypsin-like protease activity is increased in Hep 3B cells treated with camptothecin. Hep 3B cells were incubated in the presence of 1 μM camptothecin, 5 μM etoposide, or diluent for 48 h. Some camptothecin-treated cells were also incubated in the presence of 100 μM TLCK. Intracellular trypsin-like protease activity was quantified using the fluorogenic substrate Z-Val-Leu-Lys-CMAC as described in Methods.

failure of the topoisomerase II poison etoposide to induce apoptosis does not appear to be explained by the absence of topoisomerase II enzyme or the failure of etoposide to stabilize covalent topoisomerase II–DNA complexes. Indeed, topoisomerase II was detectable in Hep 3B cells by immunoblotting; and etoposide-stabilized protein-linked DNA strand breaks (presumably reflecting topoisomerase II-DNA complexes) were readily detectable by alkaline elution 30 min after addition of etoposide to the cells. Thus, the observation that topoisomerase I inhibitors selectively induce apoptosis in Hep 3B cells raises the possibility that topoisomerase I-DNA complexes might be processed differently from topoisomerase II-DNA complexes in these cells.

ICE-like and CPP32-like proteases have been implicated as participants in the execution phase of apoptosis in many cell types (31, 49, 66, 67). Moreover, apoptotic cleavage of PARP into its 24- and 89-kD signature fragments by ICE family proteases has been observed in all cell types previously examined (29). Based on these observations, investigators have even used PARP cleavage as a criterion to identify new proapoptotic proteases of the ICE family. However, in the present study, Hep 3B cells were observed to undergo apoptosis without an increase in PARP cleavage. Moreover, camptothecin-induced apoptosis in Hep 3B cells occurred without any detectable increase in ICE-like or CPP32-like protease activity. To our knowledge, this is the first cell system that appears to undergo apoptosis without activation of CPP32-like ICE family proteases and without any evidence of apoptosis-associated PARP cleavage.

We have previously suggested that a TLCK-inhibitable, trypsin-like protease activity contributes to bile salt-induced apoptosis of rat hepatocytes (39). Based on the reasoning that malignant hepatocytes might use the same effectors as normal hepatocytes, we assessed the possibility that a trypsin-like protease activity might be elevated in apoptotic Hep 3B cells and that TLCK might inhibit camptothecin-induced apoptosis of these cells. Using a novel substrate that detects peptidase activity in situ, we observed a threefold increase of intracellular trypsin-like TLCK-inhibitable protease activity during camptothecin-induced apoptosis of Hep 3B cells. These data suggest that a yet-unidentified protease with tryptic activity (recognition of basic amino acids in the P1 position) contributes, in part, to apoptosis of hepatocellular carcinoma cells. However, the data also suggest that TLCK was more effective in inhibiting the protease activity (Fig. 8) than it was in preventing apoptosis (Fig. 6). These observations suggest that additional effector mechanisms might contribute to apoptosis in this model or that minimal protease activity might be sufficient for the apoptotic cascade to be executed. The precise role of this putative protease in apoptosis will require its identification as well as manipulation of its activity using molecular biology approaches (i.e., antisense techniques, dominant-negative approaches, or a knock-out animal).

Although we have described the protease contributing to camptothecin-induced apoptosis of Hep 3B cells as trypsin-like, we did not observe cleavage of PARP in our studies despite the fact that PARP has three established trypsin cleavage sites (29). There are several potential explanations as to why the trypsin-like protease might not cleave PARP. First, although the putative trypsin-like protease recognizes a basic amino acid in the P1 position like trypsin, it might have different substrate specificity than trypsin itself and not be able to recognize the classical trypsin cleavage sites in PARP. Second, the compartmentalization of the protease within the cell might be different from PARP; and the protease might not have access to the protein (e.g., cytoplasmic vs nuclear localization). Finally, the classification of the protease as a trypsin-like protease based on its recognition of a small, tripeptide fluorogenic substrate might not reflect its endoproteinase activity on large proteins with complex tertiary structures.

Although we have also demonstrated that camptothecin induces apoptosis in Hep G2 cells (data not shown), further studies using numerous human HCC cell lines and clinical HCC specimens will be required to determine whether the observation that camptothecin selectively induces apoptosis in Hep 3B cells can be widely extrapolated to human HCCs. Hep 3B cells were used for the present studies because, like many HCCs worldwide, they are hepatitis B surface antigen positive and lack functional p53 (15, 34). Moreover, additional studies (not shown) indicate that Hep 3B cells, like most human HCCs, lack bcl-2, an inhibitor of apoptosis (68). Thus, the phenotype of these cells appears to be similar to many human HCCs. To the extent that the observations in Hep 3B cells are representative of HCC in general, these results suggest that the transduction and execution pathways for apoptosis might differ between HCC and leukemia cells or other malignancies. First, HCCs might use a unique pathway that does not depend on ICE-like proteases during the execution phase of apoptosis. Second, as illustrated by the observations with etoposide, specific apoptotic transduction pathways might be inactive in HCC cells. These observations might provide insight into the resistance of HCC to many chemotherapeutic agents.

The present observations also have potential implications for the treatment of human HCC by topoisomerase I-directed agents. Although the camptothecin concentrations required to obtain maximal apoptosis in Hep 3B cells are severalfold higher than those obtainable in humans, the concentration of pyrazoloacridine used in the present study is in the concentration range achievable in humans (69). Furthermore, locoregional therapies such as hepatic artery infusion or hepatic ar-

tery chemoembolization (70) can be used in treating HCC. These locoregional therapies permit exposure of the tumor to high concentrations of the drug while minimizing systemic effects. The observation that high concentrations of topoisomerase I–directed agents induce apoptosis in hepatoma cells suggests that trials of locoregional therapy with these agents should be considered in HCC.

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