Topoisomerase Inhibitor Camptothecin Sensitizes Mouse Hepatocytes *In Vitro* and *In Vivo* to TNF-Mediated Apoptosis

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Topoisomerases are nuclear enzymes that maintain and modulate DNA structure. Inhibitors of topoisomerases like camptothecin (CPT), etoposide, and others are widely used antitumor drugs that interfere with transcription, induce DNA strand breaks, and trigger apoptosis preferentially in dividing cells. Because transcription inhibitors (actinomycin D, galactosamine, α-amanitin) sensitize primary hepatocytes to the cytotoxic action of tumor necrosis factor (TNF), we reasoned whether topoisomerase inhibitors would act similarly. CPT alone was not toxic to primary cultured murine hepatocytes. When incubated with CPT, murine hepatocytes displayed an inhibition of protein synthesis and were thereby rendered sensitive to apoptosis induction by TNF. Apoptosis was characterized by morphology (condensed/fragmented nuclei, membrane blebbing), caspase-3-like protease activity, fragmentation of nuclear DNA, and late cytolysis. Hepatocytes derived from TNF receptor-1 knockout mice were resistant to CPT/TNF-induced apoptosis. CPT treatment completely abrogated the TNF-induced NF-kappa B activation, and mRNA expression of the antiapoptotic factors TNF-receptor associated factor 2, FLICE-inhibitory protein, and X-linked inhibitor of apoptosis protein was also inhibited by CPT. The caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (zVAD-fmk) and benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-chloromethylketone (zDEVD-fmk), as well as depletion of intracellular ATP by fructose prevented CPT/TNF-induced apoptosis. *In vivo*, CPT treatment sensitized mice to TNF-induced liver damage. In conclusion, the combination of topoisomerase inhibition and TNF blocks survival signaling and elicits a type of hepatocyte death similar to actinomycin D/TNF or galactosamine/TNF. During antitumor treatment with topoisomerase inhibitors, an impaired immune function often results in opportunistic infections, a situation where the systemic presence of TNF might be critical for the hepatotoxicity reported in clinical topoisomerase inhibitor studies. (HEPATOLOGY 2004;39:1311–1320.)

Topoisomerases are important nuclear enzymes that regulate DNA tertiary structure by mediating transient, protein-shielded DNA breaks. Topoisomerases are involved in DNA replication, repair, recombination, and in transcriptional regulation. Thus, their activity is particularly required during mitosis to facilitate DNA re-organisation. Type I topoisomerases cut and pass single strands of DNA, whereas type II enzymes cut and pass double-stranded DNA.

Topoisomerase II inhibitors (etoposide [ETP], doxorubicin) and the topoisomerase I inhibitor camptothecin (CPT) and derivatives are in clinical use or clinical trials as chemotherapeutics.[1] By covalently trapping topoisomerases on DNA, the compounds preferentially target dividing cells that require high topoisomerase activity. Under topoisomerase inhibition, DNA strand breaks occur during mitosis, which triggers premature termination of replication and inhibits

Abbreviations: CPT, camptothecin; TNF, tumor necrosis factor; ETP, etoposide; ActD, actinomycin D; NF-κB, nuclear factor-kappa B; TNFR1, tumor necrosis factor-receptor 1; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; X-IAP, X-linked inhibitor of apoptosis protein; FLIP, FLICE-inhibitory protein; TRAF, TNF-receptor associated factor; TPC, topothecan; ALT, alanine aminotransferase; FADD, Fas-associated death domain protein; OD, optical density.

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transcription, eventually leading to apoptosis (previously reviewed[2,3]).

CPT was identified as the antitumor component of *Camptotheca acuminata*.[4,5] Although the molecular target for CPT had been established early, the pathways responsible for its cell death-inducing activity remained controversial. The following mechanisms have been ascribed (reviewed previously[6,7]): due to DNA-damage, CPT activates p53 and induces downstream pro-apoptotic mechanisms.[8] CPT treatment directly up-regulates CD95/Fas and TRAIL receptor 2,[9,10] and CPT-triggered apoptosis requires *de novo* synthesis of unknown pro-apoptotic molecules in certain experimental systems.[11] CPT can also induce cell death independent of p53,[12,13] and either dependent on or independent of cell cycle progression[14–17] or caspase activation.[11,12,14,15,18] Thus, it appears that CPT can trigger a plethora of pathways leading to apoptosis, which differ from cell to cell.

By inhibiting hepatic transcription, the hepatotoxins galactosamine,[19,20] α-amanitin,[21] and actinomycin D (ActD)[22] sensitize hepatocytes 10,000-fold against the cytotoxic action of tumor necrosis factor (TNF).[22–24] Because the clinically relevant topoisomerase inhibitor CPT has been shown to block transcription under cell-free conditions,[25] we reasoned whether CPT could inhibit hepatocyte transcription and thus sensitizes hepatocytes to TNF. Also, most studies on apoptosis induction by CPT were done in mitotically active tumor cells, which prompted us further to study the effect of CPT on nondividing hepatocytes. We demonstrate that CPT restrains hepatocyte transcription, down-regulates the expression of anti-apoptotic factors, and blocks TNF-induced nuclear factor-kappa B (NF-κB) activation, thereby sensitizing hepatocytes to TNF-induced apoptosis. This effect is a likely explanation for the hepatotoxicity observed in clinical studies using topoisomerase inhibitors.[26,27]

Materials and Methods

Reagents. Topoisomerase inhibitors were from Sigma (Deisenhofen, Germany), caspase inhibitors from Bachem (Bubendorf, Switzerland), and mu-TNF was provided by Dr. G.A. Adolf (Bender & Co., Vienna, Austria). Liver perfusion, hepatocyte isolation medium, Dulbecco's modified Eagle's medium/Ham's F12 medium, and ITS (insulin/transferrin/selenium mixture) were from Gibco (Invitrogen, Carlsbad, CA). Propidium iodide was from Molecular Probes (Eugene, OR). All other reagents were bought from Sigma (Deisenhofen, Germany).

Animals and* In Vivo *Experiments. Pathogen-free male BALB/c, C57BL/6, and FVBN mice were provided by animal facilities of the University of Konstanz and the IMCB Singapore, and tumor necrosis factor-receptor 1 $(TNFR1)^{-/-}$ C57BL/6 mice were a gift of H. Bluethmann (F. Hoffmann-La Roche AG, Basel, Switzerland).[28] Animals were maintained under standard conditions and received humane care in concordance with the National Institutes of Health (NIH) guidelines and with the legal requirements in Germany and Singapore. CPT (50 mg/kg) was pre-dissolved (0.1 N NaOH, pH neutralized, end volume: 500 μL/25 g mouse) and injected intraperitoneally in endotoxin-free saline. TNF was injected intravenously (saline plus 0.1% human serum albumin). After 8 hours, mice were euthanized by intravenous injection of 150 mg/kg pentobarbital plus 0.8 mg/kg heparin. Blood was withdrawn by cardiac puncture and centrifuged (5 min, 14,000*g*, 4°C) to obtain plasma. Liver damage was assessed by measuring plasma alanine aminotransferase activity with an EPOS 5060 analyzer (Netheler & Hinz, Hamburg, Germany) according to Bergmeyer.[29] For histology, livers were perfused with cold buffer (50 mM phosphate, 120 mM NaCl, 10 mM EDTA, pH 7.4) and excised. Specimens were cut into 1-mm thick slices and fixed in 4% buffered formalin and embedded in paraffin. Sections (5 μm) were cut, stained with hematoxylin-eosin and photographed at 100-fold magnification.

Culture of Primary Murine Hepatocytes and AML-12 Cells. Hepatocytes were isolated by the two-step collagenase perfusion method of Seglen[30] using Gibco liver perfusion and Gibco liver digest media, and cultured as previously described.[23,31] Cells were plated in collagen-coated 24-well plates (8×10^4 hepatocytes/well, RPMI 1640, 10% fetal calf serum [FCS]) or in 70-mm^2 flasks (6×10^5 hepatocytes/flask), and were allowed to adhere for 3 hours before medium exchange against FCS-free RPMI 1640. AML-12 mouse hepatocytes were maintained in 70-mm^2 flasks, and cultured on 24-well plates (10^5 cells/well) in Dulbecco's modified Eagle's medium/Ham's F12 medium (supplemented with 10% FCS, ITS, an insulin/transferrin/selenium mixture) as previously described.[32,33] Apoptosis was induced by topoisomerase inhibitors given in combination with recombinant murine TNF (100 ng/mL, +30 min). Incubations were carried out at 37°C in an atmosphere composed of 5% CO_2, 40% O_2, and 55% N_2. Cytotoxicity was quantitated by measurement of lactate dehydrogenase (LDH[23,29]). For the N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-afc) cleavage assay, cells were lysed (25 mM Hepes; pH 7.5; 5 mM $MgCl_2$; 1 mM EGTA; 0.1% Triton X-100; 1 mM PEFA block; and 1 μg/mL each of pepstatin, leupeptin, and aprotinin), centrifuged (15 min, 13,000*g*, 4°C), and supernatants were frozen at −80°C. Samples for the DNA fragmentation enzyme-linked im-

munosorbent assay (ELISA) were obtained by lysis of hepatocytes (45 min at 4°C in 20 mM Tris pH 8, 10 mM EDTA, 0.1% Triton X-100) and subsequent centrifugation (15 min, 13,000*g*, 4°C).

Determination of DEVD-afc Cleavage Activity, DNA Fragmentation, and Protein Synthesis. The cleavage assay specific for caspase-3-related proteases was carried out exactly as previously described.[31] DNA fragmentation was measured by quantification of nucleosome-bound DNA using an ELISA (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instruction. The cytosolic fraction prepared as described above was diluted (300 hepatocytes/sample), and the signal of the untreated control was set to 100%. Protein synthesis was determined by the ^{3}H leucine incorporation method precisely as previously described.[23]

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from 70-mm^2 flasks using a nuclear extraction kit (Panomics, Redwood City, CA). Double-stranded NF-κB consensus oligonucleotide (sc-2505, Santa Cruz Biotechnology, CA) was 5′-endlabeled with γ^{32}P-ATP using T4 polynucleotide kinase (Promega, Heidelberg, Germany). Nuclear proteins (10 μg) were incubated at room temperature in a 25 μL reaction volume for 20 minutes (25 mM HEPES pH 7.5, 10^6 cpm radiolabeled oligonucleotide probe, 2 μg poly[dIdC], 20% glycerol, 12 mM $MgCl_2$, 100 mM KCl_2, 0.1% NP40, 1 mM DTT). Nucleoprotein-oligonucleotide complexes were resolved by nondenaturing polyacrylamide gel electrophoresis (5%), the gel was dried for 2 hours at 80°C, and autoradiographed at −80°C for 8 hours. The specificity of the DNA-protein complex was confirmed by competition with excess of unlabeled NF-κB sequence versus mutant oligonucleotide (sc-2511). The densitometry analysis for EMSA and the reverse transcriptase polymerase chain reaction (RT-PCR) data was done with a BioRad (Hercules, CA) GS-700 Imaging Densitometer and Multi-Analyst software.

Semi-quantitative RT PCR Analysis. Total RNA was extracted with Tri RNA isolation reagent (Molecular Research Center, New York, NY), and RT-PCR analysis was performed using a one-step RT-PCR kit following the manufacturer's recommended protocols (Qiagen, Hilden, Germany). Total RNA (2 μg) was used in each reaction, and the primers used for RT-PCR reactions were as follows (all 5′ to 3′): actin-fwd: gtgacataaaggagaagctgtgctatg/rev: gaagcatttgcggtggacgatggaggg; TNFR1-fwd: gtcccttctcttggtgaccgggag/rev: ggatagaaggcaaagacctagcaag; Bcl-X_L-fwd: gcagtgaagcaagcgctgagagaggc/rev: gtcccagccgccgttctcctggatcc; X-linked inhibitor of apoptosis protein (X-IAP)-fwd: gatagatggcagtatggagactcag/rev: gctgctccttgttaactgaggatg; FLICE-inhibitory protein (FLIP)-fwd: gtgcacagcagacgtatctcacttg/rev: cactatacaccatacaataacttg; TNF-receptor associated factor (TRAF)2-fwd: gtgtcctgcatgtaaaggcctggtccg/rev: gtgctgccggctacaagcctctgcag. The cycling conditions were: 50°C/30 minutes, 95°C/15 minutes, 28 cycles 95°C/30 seconds, 58°C/1 minute, 72°C/1 minute; final extension: 72°C/5 minutes.

Propidium Iodide Staining. Hepatocytes were treated on collagen-coated glass slides, fixed with ice-cold 80% methanol, and incubated with 100 μg/ml ribonuclease A (10 min, 37°C). Propidium iodide (20 μg/ml in PBS) was added (10 min at 37°C). After washing, specimens were mounted on glass slides. Pictures were recorded using a Zeiss AxioCam CCD camera fixed on Zeiss Axioplan microscope.

Statistics. Data are given as means ± SD (*in vitro* experiments) or ± S.E.M. (*in vivo* experiments). Statistical differences were determined using GraphPad Instat© by ANOVA followed by Dunnett multiple comparison test of the control versus other groups. *P* less than .05 was considered significant. All graphs show triplicate determinations of one representative of at least three experiments.

Results

CPT Sensitizes Primary Mouse Hepatocytes to TNF-dependent Apoptosis. In an initial set of experiments, we investigated whether CPT alone or in combination with death receptor agonists can induce hepatocyte death. We observed that when incubated with CPT alone, primary murine hepatocytes did not undergo cell death (Fig. 1A). In contrast, CPT in combination with a non-toxic concentration of TNF (CPT/TNF) killed about 75% of cells within 24 hours, and we determined the EC_{50} value for CPT given in combination with TNF to be 13 μM (Fig. 1A). We also checked whether CPT would sensitize hepatocytes to apoptosis induced by an activating α-CD95 antibody. Here, we found a comparatively lesser sensitization, *i.e.,* a 2-fold increase in overall death (not shown). This is accordance with previous results for the combination of ActD and cycloheximide with α-CD95.[31,34]

With regard to the mode of cell death, CPT/TNF clearly induced apoptosis: DNA fragmentation was detectable at a very early time point (6 h; Fig. 1B); we observed extensive membrane blebbing, shrinkage of the cells, and the occurrence of apoptotic bodies, as well as nuclear changes typical for apoptosis (stage II chromatin condensation: lumped, half-moon shaped dense chromatin, Fig. 1C). Time course experiments revealed that LDH leakage (secondary necrosis) occurred from 16 hours onwards (Fig. 2A), clearly preceded by the activity

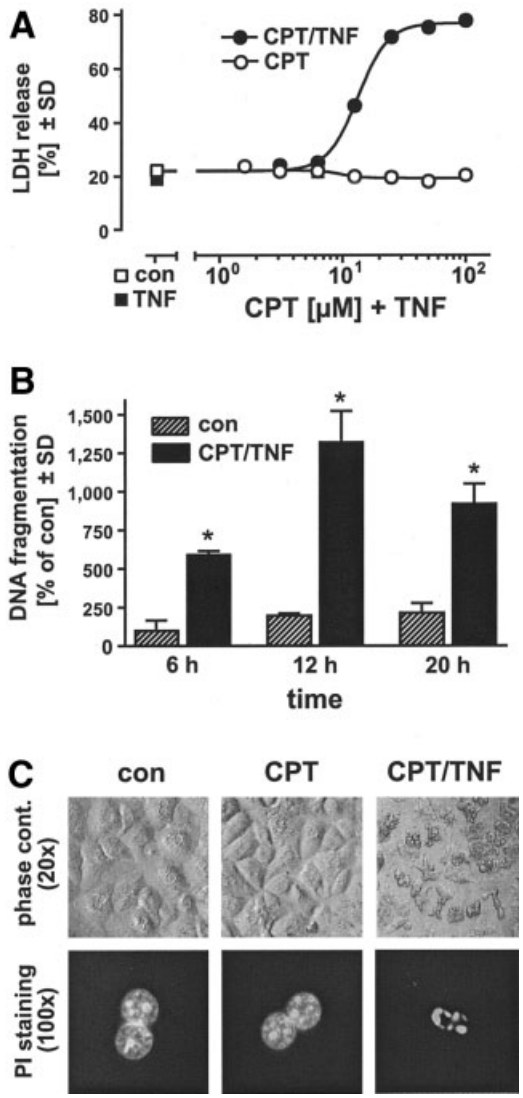

Fig. 1. Induction of hepatocyte apoptosis by CPT plus TNF. Murine hepatocyte cultures were incubated with CPT (100 μM or as indicated) and TNF (100 ng/ml, +30 min). (A) After 24 hours, cytotoxicity was determined by LDH release measurement. (B) The DNA fragmentation induced by CPT/TNF was determined by a DNA-oligosome-specific enzyme-linked immunosorbent assay (untreated control = 100%; * = *P*-value $<$ .01 vs. untreated, ANOVA/Dunnett multiple comparison test). (C) The cellular morphology (20$\times$ magnification, phase contrast) and the morphology of individual nuclei (100$\times$ magnification, propidium iodide staining) were reviewed at 16 hours. CPT, camptothecin; TNF, tumor necrosis factor; LDH, lactate dehydrogenase.

of caspase-3-like proteases detectable at 8–12 hours and peaking at 16 hours (Fig. 2B). This shows that overall cell death induction by CPT/TNF is very similar to ActD/TNF-induced apoptosis in mouse hepatocytes.[23]

Sensitization to TNF by CPT in AML-12 Hepatocytes and by Etoposide/Topothecan in Primary Mouse Hepatocytes. Next, we asked whether this sensitization observed is restricted to non-dividing hepatocytes, and whether topoisomerase inhibitors other than CPT would display this effect. We used non-transformed hepatocyte AML-12 cells, which are mitotically active in cell culture and die by apoptosis after ActD/TNF stimulation with very similar kinetics when compared to primary mouse hepatocytes.[32,33] Again, we observed that CPT sensitized cells to TNF-triggered apoptosis (Fig. 3A), showing that this effect occurs regardless of mitotic activity.

We then examined whether apart from CPT, other topoisomerase inhibitors could sensitize primary mouse hepatocytes to TNF-mediated apoptosis. The topoisomerase inhibitors ETP and topothecan (TPC) also induced apoptosis when given in combination with TNF (Fig. 3B, caspase-3-like activity as a robust apoptosis marker is shown). Thus, several topoisomerase inhibitors have the ability to sensitize resting as well as mitotically active hepatocytes to TNF-triggered apoptosis.

Inhibition of Protein Biosynthesis and TNFR1 Dependency of CPT/TNF-induced Apoptosis. It was then of interest whether a concentration of CPT that induced apoptosis when given in combination with TNF would significantly inhibit gene expression. We determined overall protein biosynthesis by ^{3}H leucine incorporation

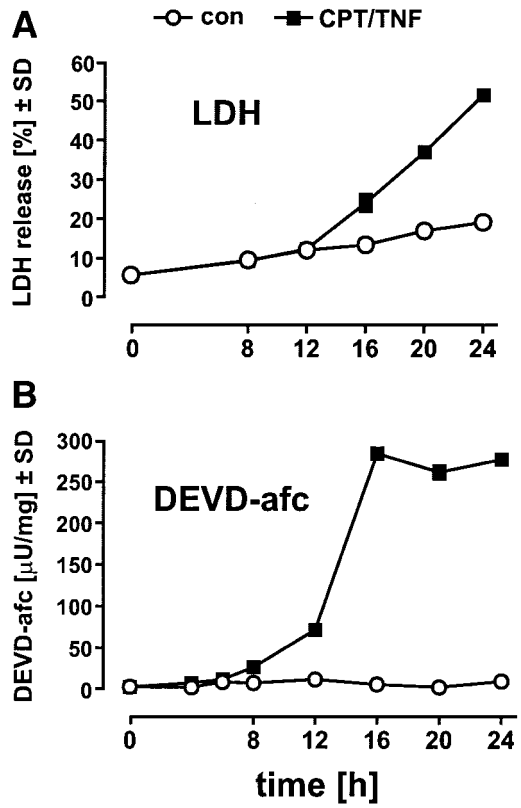

Fig. 2. Time course of CPT/ TNF-induced hepatocyte apoptosis. The time course of (A) LDH release and (B) caspase-3-like activity (DEVD-afc cleavage assay) was monitored after treatment of cells with CPT/TNF (incubation conditions as in Fig. 1). CPT, camptothecin; TNF, tumor necrosis factor; LDH, lactate dehydrogenase; DEVD-afc, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin.

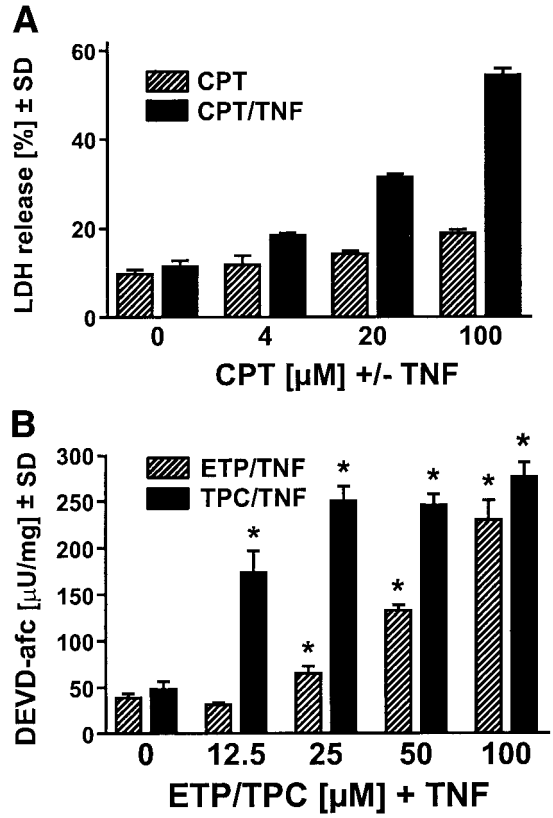

Fig. 3. Sensitization by CPT against TNF in AML-12 cells and by etoposide and topothecan in primary hepatocytes. (A) The mouse hepatocyte cell line AML-12 has been used to reproduce cell-death induction by CPT/TNF (incubation conditions as in Fig. 1). (B) Caspase-3-like activity induced by the topoisomerase inhibitors ETP and TPC in the presence of TNF (100 ng/ml); 12 hour incubation time; * = *P*-value < .01 versus untreated (ANOVA/Dunnett multiple comparison test). CPT, camptothecin; TNF, tumor necrosis factor; ETP, etoposide; TPC, topothecan.

and found that CPT inhibited it in a concentration-dependent manner by up to 75% (Fig. 4A). This range of inhibition was comparable to that induced by ActD (400 ng/ml, not shown, and in our previous study[23]), and apparently more than 70% inhibition of transcriptional activity is required to sensitize hepatocytes against apoptosis induction by TNF. Notably, the concentration of CPT inhibiting gene overall expression is in the micromolar range, a concentration frequently applied in studies on CPT-induced death of tumor cells.[9-18]

We further studied whether activation of TNFR1 is required for CPT/TNF-induced hepatocyte apoptosis. Using hepatocytes derived from TNFR1 knockout mice, we observed that the cytotoxicity of CPT/TNF was completely dependent on the presence of TNFR1 (Fig. 4B), which again is consistent with what has been reported for the ActD/TNF model.[24] Conclusively, topoisomerase inhibitors when given in micromolar concentrations can halt hepatocyte gene expression, thereby rendering the cells sensitive to the cytotoxic effect of TNF by activating TNFR1.

CPT Down-regulates mRNA Expression of TRAF2, X-IAP and FLIP and Inhibits TNF-induced NF-κB Activation. We further addressed the mechanism of the sensitizing effect exerted by CPT. Because CPT has been described to up-regulate death receptors,[10] we performed RT-PCR on TNFR1 mRNA but observed no changes after treatment of hepatocytes with CPT, TNF, or CPT/TNF (Fig. 5A). Similarly, Bcl-X_L and the actin control did not display changes in mRNA expression. The anti-apoptotic factors TRAF2, X-IAP, and FLIP, which are known to be dependent on NF-κB, were sharply down-regulated as early as 1 hour after CPT treatment. Note that in our experiments for cell death induction (Figs. 1–3), TNF was given in a delayed fashion at a time when these factors are presumably greatly affected by CPT. Furthermore, the TNF-induced 3-fold up-regulation of FLIP mRNA at 3 hours was reduced when cells were CPT pre-treated (Fig. 5A).

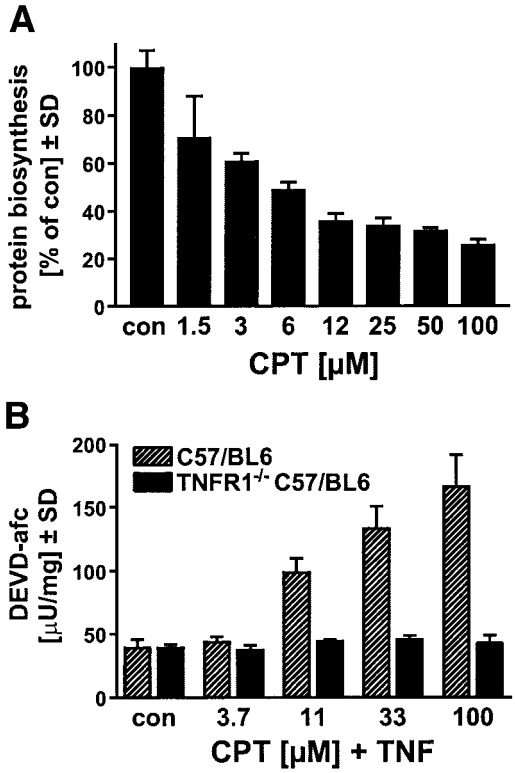

Fig. 4. CPT inhibits protein biosynthesis and sensitizes hepatocytes to TNFR1-dependent caspase activation. (A) Hepatocytes were incubated with CPT as indicated. After 18 hours, ^{3}H leucine was added for 2 hours. Incorporation of ^{3}H leucine in untreated cells was set as 100%. (B) Hepatocytes were isolated from TNFR1$^{-/-}$ C57BL/6 or C57BL/6 background control mice and incubated with CPT/TNF. After 12 hours, caspase-3-like activity was determined. TNF, tumor necrosis factor. CPT, camptothecin; DEVD-afc, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; TNFR1, tumor necrosis factor-receptor 1.

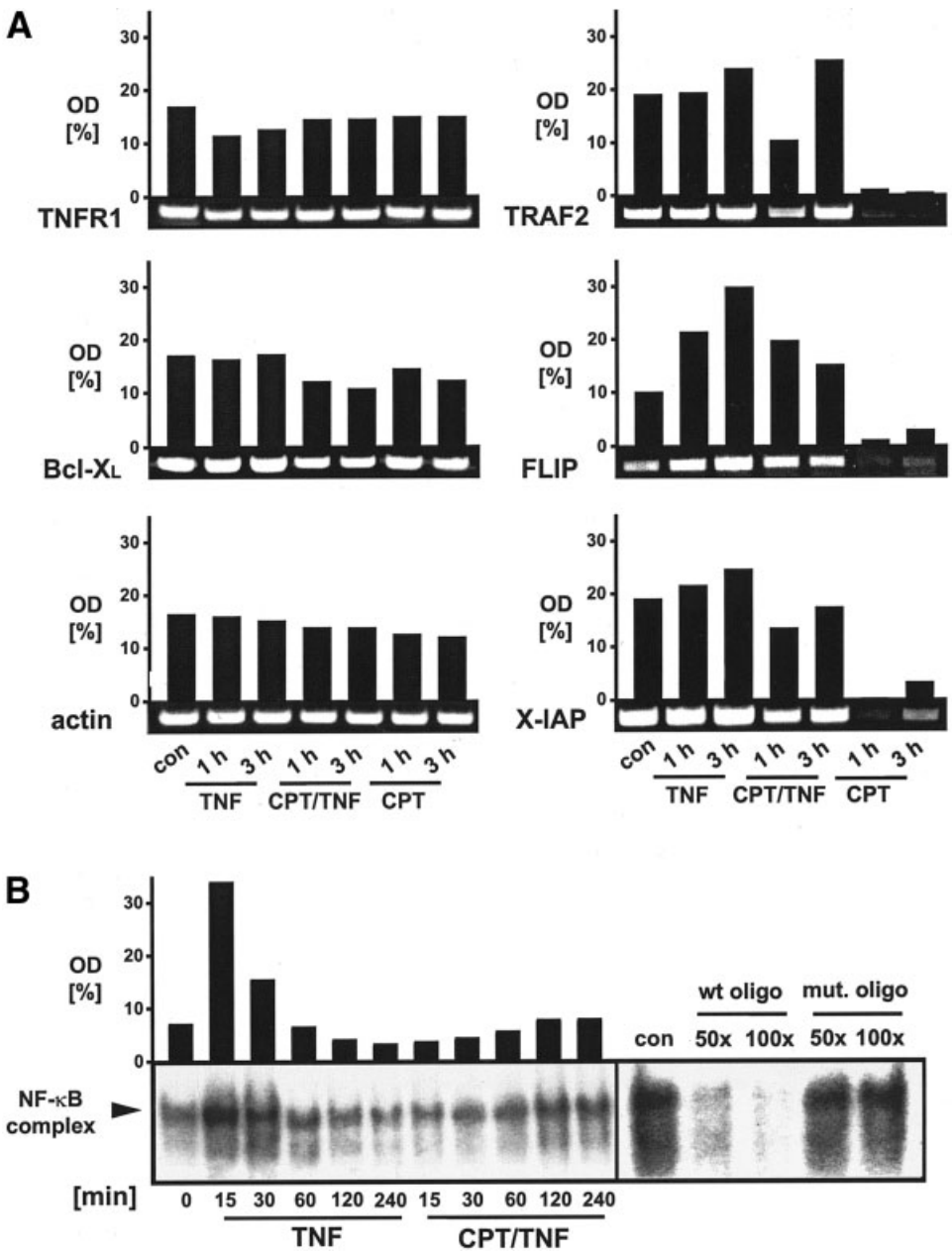

Fig. 5. Effect of CPT on gene expression and NF-kappa B activation. Murine hepatocytes were incubated with CPT and/or TNF (incubation conditions as in Fig. 1). (A) The expression of the indicated genes was determined by semi-quantitative reverse transcriptase-polymerase chain reaction at 1 and 3 hours after the indicated treatments. (B) NF-kappa B activation was monitored by electrophoretic mobility shift assay. The specificity of the binding is shown by competition with an indicated excess of unlabeled probe (wt oligo) versus mutant unlabeled probe (mut. oligo). The densitometry data shown represent % of total OD, as determined with a BioRad GS-700 Imaging Densitometer using the Multi-Analyst software. OD, optical density; TNFR1, tumor necrosis factor-receptor 1; TRAF, TNF-receptor associated factor; FLIP, FLICE-inhibitory protein; X-IAP, X-linked inhibitor of apoptosis protein; CPT, camptothecin; TNF, tumor necrosis factor.

Because NF-κB represents the major anti-apoptotic signaling pathway downstream of TNFR1, and blocking NF-κB is required to kill hepatocytes by TNF, we studied the NF-κB activation status by EMSA (Fig. 5B). We observed a more than 4-fold increase in nuclear binding of active NF-κB 15 minutes after TNF stimulation, which was abolished by CPT pre-treatment. Even at 4 hours, there was no increase in NF-κB binding as compared to the TNF-induced peak. In a similar fashion, ActD preincubation of hepatocytes prevented TNF-induced NF-κB activation (data not shown). These data imply that CPT treatment renders hepatocytes TNF-sensitive by changing the expression pattern of anti-apoptotic factors, and by blocking the pro-survival by NF-κB signaling pathway.

Modulation of CPT/TNF-induced Hepatocyte Apoptosis by Caspase Inhibition and ATP Depletion. We further revealed how CPT/TNF-induced apoptosis could be halted by known anti-apoptotic treatments. Comparing the effects of three different caspase inhibitors, we observed that the broad-range inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (zVAD)-fmk that inhibits caspases and cathepsin B[35] exerted a potent protection against CPT/TNF-mediated cell death (Fig. 6A); similarly, the more caspase-3/-7-specific inhibitor benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-chlo-

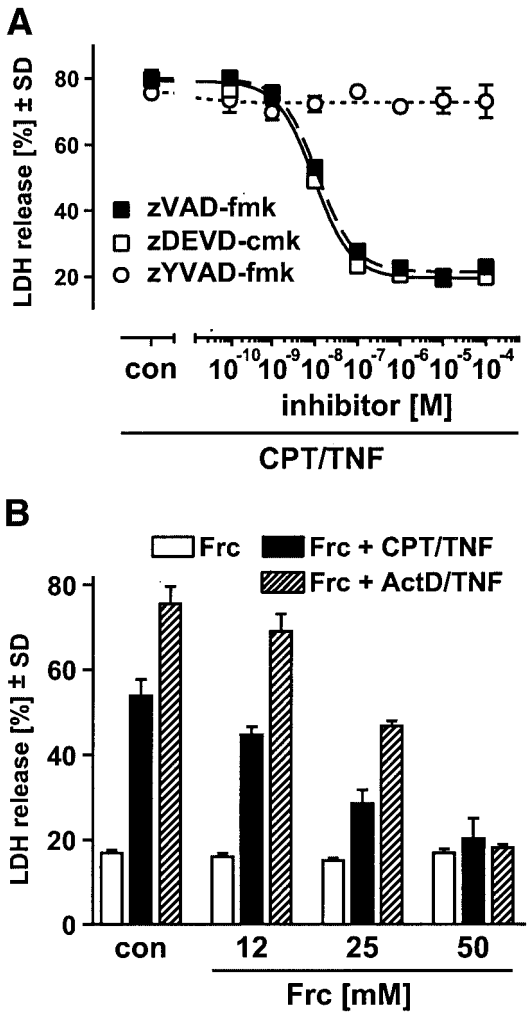

Fig. 6. Modulation of CPT/TNF-induced hepatocyte apoptosis by caspase inhibitors and ATP depletion. Murine hepatocytes were incubated with camptothecin and TNF as described for Fig. 1. After 20 hours, cytotoxicity was determined by LDH release measurement. (A) Cells were preincubated for 30 minutes with serial dilutions of caspase inhibitors as indicated. (B) Cells were pretreated for 1 hour with the ATP-depleting carbohydrate fructose with the indicated concentrations; here, both ActD and CPT were used to sensitize to TNF. LDH, lactate dehydrogenase; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone; z-DEVD-fmk, benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-chloromethylketone; z-YVAD, benzyloxycarbonyl-Tyr(OMe)-Val(OMe)-Ala-Asp (OMe)-fluoromethylketone; CPT, camptothecin; TNF, tumor necrosis factor; Frc, fructose; ActD, actinomycin D.

romethylketone (zDEVD-fmk)[36] prevented hepatocyte apoptosis. For both inhibitors, the IC_{50} was found to be 10 nM. In contrast, the caspase-1 inhibitor benzyloxycarbonyl-Tyr(OMe)-Val(OMe)-Ala-Asp(OMe)-fluoromethylketone (zYVAD-fmk) was ineffective up to 100 μM. Our data suggest that apoptosis induction by CPT/TNF is caspase-1-independent but sensitive to caspase-3/-7 inhibition, and the findings are similar to those reported for other TNFR1-dependent hepatocyte death models.[37,38]

Previously, we reported that metabolic depletion of hepatocyte ATP by ATP-depleting carbohydrates such as fructose or tagatose robustly blocked ActD/TNF-triggered hepatocyte death, while it enhanced CD95-induced apoptosis.[31] Because the metabolic pathway leading to ATP-depletion is unique for hepatocytes, it was of interest whether death induced by CPT/TNF is sensitive to this intervention in comparison to the ActD/TNF model. Pre-incubation of the cells with a phosphate-trapping sugar, fructose, prevented both ActD/TNF- and CPT/TNF-induced hepatocyte apoptosis in a similar concentration-dependent manner (Fig. 6B). It therefore appears that ATP depletion in a hepatocyte-specific way might provide a means to lower the toxicity of CPT/TNF in the liver.

Induction of Liver Damage by CPT/TNF in Mice. We finally addressed the question of whether CPT would also sensitize hepatocytes to TNF-mediated death *in vivo*. We injected five groups of Balb/c mice with combinations of CPT (50 mg/kg) and TNF (5 and 7 μg/kg). In agreement with what we had observed in the *in vitro* experiments, CPT alone did not induce hepatocyte death, but merely sensitized the liver to the toxic effects of TNF (Fig. 7A): severe damage of liver parenchymal cells, as quantified by measurement of the hepatocyte-specific enzyme alanine aminotransferase (ALT) in plasma samples, was observed 8 hours after treatment of the animals with

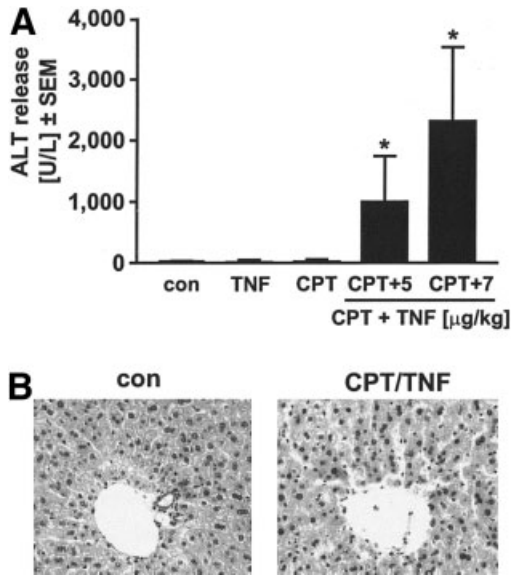

Fig. 7. Induction of hepatic damage by CPT and TNF in mice. Five groups of mice (n = 3/group) received combinations of CPT (intraperitoneally 50 mg/kg) and TNF (intravenously, injected 30 min after CPT) or were left untreated (con) as indicated. (A) After 8 hours, the amount of liver damage was determined by plasma ALT quantification (* = *P*-value < .01 vs. control animals, based on ANOVA/Dunnett multiple comparison test). (B) Liver histopathology 8 hours after CPT/TNF injection (50 mg/kg, 7 μg/kg; hematoxylin-eosin staining; original magnification ×100). ALT, alanine aminotransferase; TNF, tumor necrosis factor; CPT, camptothecin.

CPT/TNF. The histology of liver specimens from those mice (Fig. 7B) resembled that of GalN/TNF-mediated injury, including pyknotic hyperchromatic nuclei and a desertion of sinusoidal endothelial cells. At this late time point (8 h), hepatocyte necrosis and a disruption of the sinusoidal liver structure were widely observed. We also intended to investigate a possible protective effect of fructose in this *in vivo* model in analogy to GalN/TNF-mediated liver injury,[31] but this attempt failed because both fructose and CPT have to be injected intraperitoneally in large volumes, causing rapid lethality. Taken together, treatment of mice with high-dose CPT in combination with TNF can lead to massive hepatic damage due to TNFR1-mediated hepatocyte apoptosis.

Discussion

Topoisomerase inhibitors are widely used drugs in chemotherapy.[1–3] However, in some cases, severe hepatotoxic side effects were observed.[26,27] Our report shows that CPT *per se* is unable to induce hepatocyte death, but rather sensitizes hepatocytes *in vitro* and *in vivo* to TNF-induced apoptosis. We also reveal that caspase inhibitors and ATP depletion have a protective potential against CPT/TNF-triggered hepatocyte death.

In tumor cells, the cell death-enhancing synergism of topoisomerase inhibitors and TNF was noted very early,[39–42] well before TNF signaling was established. Because topoisomerase activity was found to be increased after TNF, it was suggested that topoisomerases are directly involved in TNF signaling,[39,40] or alternatively that the observed sensitization to TNF is related to DNA strand breaks.[42] Additionally, two recent papers report that topoisomerase inhibitors cause increased gene expression of death receptors, and they further describe a synergism of topoisomerase inhibitors and death receptors other than TNFR1.[43,44] Here, we show that although CPT/TNF-induced hepatocyte apoptosis depends on TNFR1, there was no further induction of TNFR1 from an already high basal expression level in our system (Fig. 5A). Overall, it appears that the earlier proposals to use TNF alongside with topoisomerase inhibitors are questionable due to the sensitization of hepatocytes against TNF (this study) and the reported hepatotoxicity in human clinical studies.[26,27]

We also addressed how CPT action and transcription inhibition interfere with the TNF signal transduction. An apoptotic and an anti-apoptotic pathway characterize TNFR1 signaling (reviewed previously[45]). Upon TNF binding, TNFR1 trimerizes, which induces binding of the adapter proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) to the intracellular part of the receptor. After binding to FADD, upstream caspase-8 becomes activated, triggering downstream caspases. This pro-apoptotic proteolytic pathway is regulated by an anti-apoptotic pathway mediated by NF-κB, downstream of TRAF2. We observed both a strong down-regulation of TRAF2 as well as a prominent absence of TNF-induced NF-κB activation when CPT was present (Fig. 5). These data are in good agreement with a recent study of Valente *et al.*[46] showing that the early peak of NF-κB activation by TNF is inhibited by CPT in ovarian cells. Thus, CPT pretreatment sensitizes hepatocytes to TNF killing presumably by down-regulating TRAF2 expression, which in turn blocks NF-κB activation, as well as by hampering the expression of NF-κB-dependent genes such as FLIP and X-IAP.

We observed discrepancies between experimental systems with regard to sensitization to TNF. Using a standard collagenase (Sigma C-5138), CPT alone was toxic and TNF merely enhanced apoptosis (20 h: con, 23.4%; CPT, 63.3%; CPT/TNF, 65.9%). This was in contradiction to the synergistic effect *in vivo* (Fig. 7) and in the hepatocyte line AML-12 (Fig. 3A). On the other hand, hepatocytes have been reportedly killed by exposure to CPT or galactosamine alone.[47,48] These disparities may be caused by contaminating activated Kupffer cells in the hepatocyte culture or due to the activation of liver-resident Kupffer cells producing TNF during the liver perfusion. We therefore purified the hepatocytes using a multistep Percoll gradient,[49] a method that excludes any macrophage contamination. Again, CPT alone induced apoptosis of hepatocytes (20 h: con, 25.5%; CPT, 54.9%; CPT/TNF, 54.5%). Comparing different collagenases, we found that a collagenase-dispase medium from Gibco gave yields of hepatocytes that reproducibly tolerated high concentrations of CPT as well as other transcriptional inhibitors. Apparently, the reagents used for the liver digestion procedure must be carefully considered as determinants for hepatocyte sensitivity to apoptosis-stimulating stimuli.

Patients undergoing chemotherapy frequently have necrotic tissue lesions or are compromised in their immune status. This renders them prone to infections, leading to an inflammatory state that causes further necrotic tissue lesions (Fig. 8). Under such conditions, TNF can be released systemically by monocytes and macrophages, and it is also intriguing that a CPT analogue (irinotecan) was shown to directly induce TNF production in monocytes.[50] Because the sensitization of hepatocytes against TNF action by transcriptional inhibition is particularly potent when compared to other cell types,[23] TNF in such a scenario would preferentially induce hepatotoxicity. In fact, severe hepatotoxic effects of potent topoisomerase

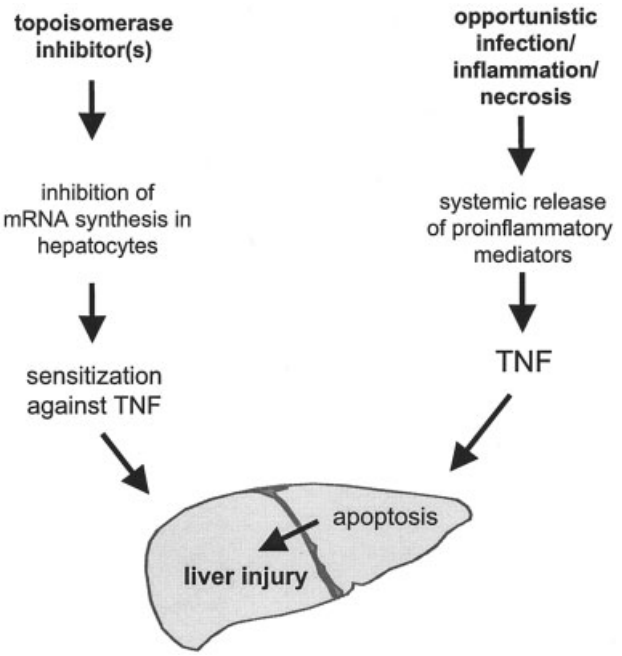

Fig. 8. Proposed sequence of events causing acute hepatitis during topoisomerase inhibitor treatment. Our study indicates that topoisomerase inhibitors can sensitize hepatocytes towards the cytotoxic action of TNF by transcriptional inhibition. When TNF is systemically released under this condition due to an opportunistic infection or tissue necrosis, fulminant liver may occur. TNF, tumor necrosis factor.

inhibitors have been reported in two clinical studies.[26,27] Therefore, a desirable strategy is to block TNF-mediated adverse effects of CPT selectively in the liver. Fructose, for instance, neither depletes ATP nor does it halt apoptosis in cell lines, hepatoma cells,[31] or AML-12 cells. Hepatocyte-specific metabolic pathways interfering with apoptosis-inducing pathways may be exploited in the future to differentially modulate responses of tumor cells and hepatic parenchymal cells.

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