

Induction of Neuronal Apoptosis by Camptothecin, An Inhibitor of DNA Topoisomerase-I: Evidence for Cell Cycle-independent Toxicity

Erick J. Morris and Herbert M. Geller

Department of Pharmacology, University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Abstract. Camptothecin is an S-phase-specific anticancer agent that inhibits the activity of the enzyme DNA topoisomerase-I (topo-I). Irreversible DNA double-strand breaks are produced during DNA synthesis in the presence of camptothecin, suggesting that this agent should not be toxic to nondividing cells, such as neurons. Unexpectedly, camptothecin induced significant, dose-dependent cell death of postmitotic rat cortical neurons *in vitro*; astrocytes were more resistant. Aphidicolin, an inhibitor of DNA polymerase α , did not prevent camptothecin-induced neuronal death, while death was prevented by actinomycin D and 5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole as well as cycloheximide and anisomycin, inhibitors of RNA and protein synthesis, respectively. Camptothecin-induced neuronal death was apoptotic, as characterized by chromatin condensation, cytoplasmic shrinking,

plasma membrane blebbing, and fragmentation of neurites. DNA fragmentation was also confirmed by the use of the *in situ* DNA end labeling assay. In addition, aurintricarboxylic acid, an inhibitor of the apoptotic endonuclease, partially protected against camptothecin-induced neuronal death. The toxicity of stereoisomers of a camptothecin analogue was stereospecific, demonstrating that toxicity was a result of inhibition of topo-I. The difference in sensitivity to camptothecin between neurons and astrocytes correlated with their transcriptional activity and level of topo-I protein expression. These data indicate important roles for topo-I in postmitotic neurons and suggest that topo-I inhibitors can induce apoptosis independent of DNA synthesis. We suggest a model based on transcriptionally mediated DNA damage, a novel mechanism of action of topo-I poisons.

PROGRAMMED cell death is an important physiological process that results in the death of ~50% of the neurons formed during embryogenesis (46). This death is normally thought to result from neuronal competition for trophic support; thus, neurons that do not find their correct targets are eliminated. Neuronal programmed cell death occurs by the process of apoptosis, characterized by chromatin condensation, endonucleolytic DNA cleavage, cytoplasmic shrinkage, plasma membrane blebbing and contraction (a term also called *zeiosis* [10]), fragmentation of neurites, and formation of apoptotic bodies (30). Apoptosis is an “active” cellular process in which the cell “commits suicide” and is distinct from necrosis, which is characterized by lysis or disintegration of the cell, a “passive” form of cell death.

Much of our understanding of the biochemical and molecular events associated with neuronal apoptosis has come from the study of cultured central and peripheral neurons as well as work from cell lines which exhibit neu-

ronal properties. The typical paradigm for apoptotic neuronal death uses trophic factor-dependent cells in which trophic support is withdrawn and the subsequent cell death is studied. In particular, the study of sympathetic neurons, whose survival is dependent upon NGF *in vitro* and *in vivo*, has contributed a significant amount of information regarding the biochemical profile of apoptotic neuronal death (15). One key characteristic of this profile is that after NGF withdrawal neurons begin to die by apoptosis and this cell death can be prevented with inhibitors of RNA and protein synthesis (37). These data suggest that after NGF deprivation, an active genetic program is initiated which results in subsequent neuronal death. These data also suggest that trophic factors act by repressing an endogenous death pathway as opposed to stimulating a cell survival pathway. Currently, however, the biochemical and genetic events involved in the apoptotic pathway in neurons remain poorly understood.

Although apoptotic cell death occurs physiologically during development, neuronal apoptosis is also associated with a variety of neurodegenerative disorders and conditions of neural trauma including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, AIDS dementia, epilepsy, retinitis pigmentosa,

Address all correspondence to Erick J. Morris, Department of Pharmacology, UMDNJ—Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Tel.: (908) 235-4084. Fax: (908) 235-4073. E-mail: morris@umdnj.edu

cerebellar degeneration, and ischemic brain injury or stroke (57). This suggests that the biochemical mechanisms of physiological and pathological neuronal death may overlap. Moreover, a variety of metabolic insults can induce apoptotic death of neuronal cells (14, 51, 56), suggesting that the trophic factor-mediated repression of the death pathway can either be inhibited or overridden by other stimuli. Of particular interest is the induction of apoptosis in postmitotic neurons by antimetabolic anticancer agents. Treatment of both rat sympathetic and cerebellar neurons with the S-phase-specific inhibitor, cytosine arabinoside (AraC)¹, results in apoptosis in vitro (15, 18). In addition, some patients treated with high-dose AraC therapy for refractory leukemia develop a cerebellar toxicity syndrome characterized by the death of nondividing Purkinje neurons (63, 67). Adenosine arabinoside can also induce apoptosis in rat sympathetic neurons, although with lesser potency as compared with AraC (59). Similarly, treatment of sympathetic or cerebral cortical neurons with the G2/M-phase-specific DNA topoisomerase-II inhibitors, etoposide, teniposide, or mitoxantrone, also results in apoptotic death (42, 43, 59). Currently, it is unknown how these anticancer agents signal a terminally differentiated, postmitotic neuron to execute its apoptotic pathway or how a variety of stimuli activate a morphologically indistinguishable death response.

Camptothecin is a cytotoxic plant alkaloid originally isolated from the Chinese *Camptotheca acuminata* tree (64). This compound and its analogues are S-phase, cell cycle-specific, anticancer agents that inhibit the activity of mammalian DNA topoisomerase-I (topo-I) (for review see 7). The major function of topo-I is to relax DNA supercoiling produced by the torsional stress generated during DNA replication and transcription (for review see 65). During this process, camptothecin binds to the topo-I/DNA complex (termed the cleavable-complex [27]), resulting in a DNA single-strand break. Attempts by the cell to enter S-phase of the cell cycle, in the presence of camptothecin, result in late S- and early G2-phase arrest with the concomitant conversion of DNA single-strand breaks into potentially lethal, irreversible DNA double-strand breaks (27). This ultimately results in cell death via the apoptotic pathway (17). It is hypothesized that toxicity arising from camptothecin treatment results from the actions of the replicative machinery colliding with the cleavable-complex, resulting in DNA double-strand breaks: hence, its S-phase specificity (28).

Due to its cell cycle specificity, camptothecin would be expected to be relatively nontoxic to differentiated, postmitotic cells, such as neurons, at concentrations known to inhibit topo-I function. In this study, we have tested this hypothesis by investigating the effects of camptothecin on postmitotic cells derived from the nervous system. Unexpectedly, camptothecin induced an apoptotic cell death in postmitotic cortical neurons that was apparently mediated by the target enzyme, topo-I. Aphidicolin cotreatment did not prevent camptothecin-induced neuronal death, sug-

gesting that replication does not contribute to camptothecin neurotoxicity. However, camptothecin-induced cell death was inhibited by RNA and protein synthesis inhibitors, suggesting that active transcription and translation was necessary for toxicity. These data demonstrate that topo-I function is important for neuronal survival and that inhibition of this enzyme results in apoptosis. Moreover, since camptothecin-induced cell death of nondividing cells must be independent of DNA replication, alternative mechanisms of camptothecin-induced cell death must exist. We suggest that non-S-phase toxicity of camptothecin is dependent upon active DNA transcription, a novel mechanism of action of topo-I poisons.

Materials and Methods

Materials

Timed-pregnant Sprague-Dawley rats were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). FCS and Basal Media Eagle's (BME) were obtained from Sigma Chemical Co. (St. Louis, MO). DME was obtained from Gibco BRL (Grand Island, NY). Nylon mesh for filtering cultured cells was purchased from Tetko (Elmsford, NY). 20-S-camptothecin (referred to in the text as camptothecin) and 20-S- and 20-R- 10-11-methylenedioxy-camptothecin (MDCPT) were obtained as gifts from the laboratory of Dr. Leroy F. Liu (UMDNJ-Robert Wood Johnson Medical School). [5-³H]uridine was obtained from Amersham Life Sciences (Buckinghamshire, England). [4,5-³H]L-leucine was obtained from ICN Bio-medicals (Irvine, CA). All other reagents and tissue culture supplies were obtained from Sigma Chemical Co.

Cell Cultures

Astrocyte cultures were prepared by the method previously described (39). Cerebral cortices were removed from newborn rats (postnatal d 1–3) and placed into cold BME media with 0.02 M Na-Hepes buffer, pH 7.4 (BME-Hepes). The meningeal tissue and white matter was removed and the remaining tissue was minced into fine sections (1 mm) with sharp dissecting knives. The minced sections were incubated with 0.025% trypsin (type-II) for 30 min at 37°C. The reaction was halted with 0.025% soybean trypsin inhibitor and 0.05% DNase-I and the tissue was triturated several times through a fire-polished Pasteur pipette. The suspension was passed through sterile 40- μ m nylon mesh cloth to remove large cell aggregates and then centrifuged for 5 min at 1,000 g. The pellet was resuspended in medium composed of DME supplemented with 10% heat-inactivated FCS, NaHCO₃ (2.2 mg/ml), penicillin (50 μ g/ml), and streptomycin (50 U/ml) (DME/10% FCS). The cells were plated into poly-L-lysine-coated (100 μ g/ml) 80-cm² culture flasks (Nunc) and incubated at 37°C in 95%:5% air/CO₂ atmosphere with 100% relative humidity. When the cells become confluent (~10 d), the flasks were shaken overnight (185 rpm) on a rotary shaker to remove loosely adherent cells (neurons, O-2A progenitors, macrophages, and oligodendrocytes). Astrocytes are routinely screened via immunofluorescence techniques for the expression of GFAP as well as for the absence of oligodendrocyte and neuronal markers, and this culture method generally yields >95% GFAP-positive cells. For astrocyte monolayers, cells were plated at 3–4 \times 10⁴ cells per well in poly-L-lysine-coated 24-well plates (Falcon Plastics) in 0.5 ml of DME/10% FCS and allowed to become confluent (confluency equals approximately 7 \times 10⁴ astrocytes per 24 well). After the cells reached confluency, they were incubated with DME/2% FCS and the media was changed approximately every 3 d.

Primary dissociated cortical neurons were prepared by the method previously described (62). Briefly, timed-pregnant Sprague-Dawley rats were killed at embryonic day 17 (E17) and embryos were removed by Caesarian section. The cerebral cortex was dissected from each embryonic brain, the meningeal tissue was carefully removed, and the cortex was finely minced into sections (<0.5 mm) with sharp dissecting knives. The sections were placed into cold BME-Hepes and incubated with 0.025% trypsin (type-II) and 0.1% type-IV collagenase for 30 min at 37°C. The reaction was halted with 0.025% soybean trypsin inhibitor and 0.05% DNase-I and the tissue was gently triturated several times through a fire-polished Pasteur pipette. The cells were filtered through sterile 40- μ m mesh, resuspended in DME/10% FCS, and plated at a density of 1.5 \times 10⁵

1. Abbreviations used in this paper: AraC, cytosine arabinoside; BrdU, bromodeoxyuridine; CF, carboxyfluorescein; CFDA, 5(6)-carboxyfluorescein diacetate; DAPI, 4',6'-diamidino-2-phenylindole; TdT, terminal deoxyribonucleotidyl transferase; topo-I, DNA topoisomerase-I.

cells/well on top of a preexisting astrocyte monolayer in 24-well plates maintained in DME/2% FCS. The astrocyte monolayer provides optimal trophic support for embryonic cortical neuronal survival (62). After ~5–6 d in vitro, the mixed cultures were treated as indicated below. To ensure that neurons were postmitotic, after 5 d in vitro cultures were incubated with 10 μ M bromodeoxyuridine (BrdU) for 24 h. BrdU incorporation into DNA was evaluated immunocytochemically. Less than 0.1% of the neurons incorporated BrdU, demonstrating that 99.9% of these cells were terminally differentiated and postmitotic. Approximately 5% of the astrocytes displayed BrdU immunoreactivity, suggesting that they maintained a low degree of proliferation.

Treatment of Cultures with Pharmacological Inhibitors

Cells were treated for the experiments below as indicated. Neurons and astrocytes were incubated with pharmacological inhibitors in serum-free N2 medium or DME/2% FCS. The presence of serum had no effect on neuron or astrocyte survival after treatment with camptothecin (data not shown). Survival after treatment with the pharmacological inhibitors was compared with parallel treatment with control medium. Aphidicolin, DRB, camptothecin, and MDCPT stock solutions were dissolved in 100% DMSO. Control cultures were incubated with <0.5% DMSO solutions as vehicle control. Solutions of <0.5% DMSO were not toxic to neurons or astrocytes within the time range of the experiments described below.

CFDA Assay for Neuronal Survival

Neuronal survival was determined using an assay based upon the vital dye 5(6)-carboxyfluorescein diacetate (CFDA) (49). Mixed neuronal-astrocyte cultures were washed once with Hepes-buffered saline solution (HBS; 122 mM NaCl, 3.3 mM KCl, 1.2 mM CaCl₂, 0.4 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose, 25 mM Hepes at pH 7.3) and incubated with 10 μ M CFDA in HBS for 15 min at 37°C in 95%:5% air/CO₂ atmosphere with 100% relative humidity, protected from light. Under these conditions, both astrocytes and neurons take up and cleave CFDA to the anionic fluorophore, carboxyfluorescein (CF), by the activity of endogenous cellular esterases. The media were then aspirated and the cells were incubated with HBS for 30 min as a rinse, during which astrocytes pump CF into the media, while neurons retain it for hours. The rinse was aspirated and cells were lysed with water containing 0.1% Triton X-100. The lysate was then diluted 1:10 in 0.1 M PBS and the fluorescence measured using a Perkin-Elmer MPF-66 fluorescence spectrofluorometer (excitation λ 490 nm, emission λ 517 nm). Fluorescence intensity is linearly proportional to the number of metabolically viable neurons (49). Under control conditions, relative fluorescence for 1.5×10^5 neurons per 24 well was ~2,000 arbitrary units, while background fluorescence (identical cultures without neurons) was ~10 U. Therefore, ~99.5% of the CF fluorescence is contributed by neurons. To ensure accurate fluorometric measurements, each experiment was performed in parallel with a standard CF calibration curve.

For neuronal CF labeling, mixed cultures of neurons and astrocytes were grown on glass coverslips in 24-well plates, incubated with 10 μ M CFDA in HBS for 15 min, and rinsed as described above. After rinsing, the coverslips were inverted and mounted within a ring of vacuum grease on a glass slide in a drop of HBS. The coverslip was viewed under fluorescence epifluorescence and images captured as previously described (49).

MTT Viability Assay

Astrocyte survival was assayed by the colorimetric conversion of the yellow, water-soluble tetrazolium, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to the purple, water-insoluble formazan. This conversion is catalyzed by cellular mitochondrial dehydrogenases and therefore, can be utilized to quantify numbers of viable cells (40). Cells were assayed by a modified protocol of the procedure previously described (1). Cells were incubated with 0.1 mg/ml MTT solution in DME without phenol red for 4 h at 37°C in 95%:5% air/CO₂ atmosphere with 100% relative humidity. After the incubation, the cells were triturated into suspension and centrifuged at 8,000 *g* for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 0.8 N HCl-isopropanol solution (pH 3) containing 1% Triton X-100, which lyses the cells and solubilizes the formazan product. The absorbance of the lysate was measured at 570 nm with a microplate reader (Bio-Tek Instruments). Neurons and astrocytes totally converted the MTT formazan product within ~3–4 h. Calibration curves were conducted in parallel experiments

which demonstrated a linear correlation between MTT absorbance and cell number.

Immunohistochemistry

Cells were fixed with ice-cold ethanol/acetic acid (95:5) for 8 min at 4°C and rinsed three times in 0.1 M PBS containing 0.1% sodium azide (PBS-azide) and once in PBS-azide with 10% FCS as a blocking agent. To label neurons, coverslips were incubated with a 1:100 dilution of mouse monoclonal anti-neurofilament RT97 antibody (the generous gift of Dr. John Wood, University College, London, UK) for 30 min at 4°C. After rinsing, the coverslips were incubated with rhodamine-conjugated goat anti-mouse IgG secondary antibody (Organon-Technica Cappel) for 30 min at 4°C (1:100 dilution). For nuclear staining, cells were fixed and then incubated with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. After rinsing, the coverslips were mounted in Fluoromount G (Southern Biotechnology Associates, Inc.) and sealed with nail enamel.

Identification of DNA Topoisomerase-I Protein Expression

DNA topoisomerase-I protein levels were measured using immunoblotting techniques. Samples consisted of cellular extracts prepared from (a) E 17 rat cerebral cortex, (b) neurons cultured 2 d in vitro from E 17 rat cerebral cortex (with addition of 1 μ M 5-fluorodeoxyuridine and 1 μ M uridine at 1 d in vitro to kill dividing cells), and (c) confluent cultures of purified astrocytes cultured from newborn rat cerebral cortex. Cellular extracts were prepared by lysing cells in homogenization buffer consisting of 1% SDS, 8% sucrose, 2 mM EDTA, 3 mM EGTA, and 50 mM phosphate buffer (pH 8.0). Protein concentrations were determined using the Bio-Rad-DC protein assay. Laemmli sample buffer (5 \times) was added to the extracts and the samples were heated to 100°C for 5 min. Cell extracts (20 μ g/lane) were subjected to 10% SDS-PAGE (33) and then transferred to nitrocellulose paper (40 mA, 15 h) (60). The blots were incubated in blocking solution containing 5% dry milk (Carnation® brand) in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) for 1 h and then incubated for 2 h in a solution of primary antibody (rabbit anti-human DNA topoisomerase-I) diluted 1:1,000 in 3% dry milk/TBS. The blots were then washed three times (15 min per wash) in TBS containing 0.05% Tween 20 and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) at a 1:1,000 dilution. The blots were washed again three times as above and DNA topoisomerase-I protein was detected colorimetrically using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium development. All antibody incubations and washes were done at room temperature. Each immunoblot was run with 10 μ L/lane of kaledioscope prestained standards (BioRad) mol wt markers. Relative levels of protein expression were determined by analyzing the pixel intensity of the bands using the NIH-Image program (version 1.59). In addition, parallel SDS-PAGE gels were stained with Coomassie brilliant blue to ensure adequate protein loading of the gels. Immunoreactivity was not detected in the negative control blots in which the primary antibody was omitted.

Analysis of DNA Fragmentation

Mixed cultures were analyzed for apoptotic DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling (TUNEL) assay using the ApopTag® kit (Oncor, Gaithersburg, MD). This assay uses the ability to label free 3'-OH DNA ends that are generated by nuclear endonucleolytic cleavage of DNA which occurs during apoptosis (24). The free 3'-OH DNA ends are labeled with digoxigenin-dUTP via the terminal deoxynucleotidyl transferase (TdT) enzyme. The kit was used according to the manufacturers instructions except that it was modified for cultured cells growing on 12-mm glass coverslips. Neurons were plated at a density of 5×10^4 cell/well on preexisting astrocyte monolayers grown on poly-L-lysine-coated (100 μ g/ml) 12-mm glass coverslips in 24-well trays (Falcon). The cells were fixed with ice-cold ethanol/acetic acid (95:5) for 8 min at 4°C and rinsed three times in 0.1 M PBS. The coverslips were quenched with 30 μ L/coverslip of 2% H₂O₂ in PBS for 5 min at room temperature, rinsed three times in PBS, and incubated with 15 μ L/coverslip of 1 \times equilibration buffer for 20 min at room temperature. The cells were then incubated with 10 μ L/cm² (15 μ L/coverslip) of working strength TdT enzyme (1:2.4 TdT enzyme/reaction buffer) at 37°C in 95%:5% air/CO₂ atmosphere with 100% relative humidity. The reaction was stopped by rinsing three times in PBS and adding 15

$\mu\text{L}/\text{coverslip}$ of $1 \times$ stop/wash buffer for 30 min at 37°C in the incubator. The cells were rinsed and incubated with $25 \mu\text{L}/\text{coverslip}$ of peroxidase conjugated anti-digoxigenin secondary antibody for 30 min in the incubator. The cells were then rinsed and color developed using a 0.75% diaminobenzidine tetrahydrochloride solution and mounted on glass slides. As a positive control, cells were incubated with $1 \mu\text{g}/\text{ml}$ of DNase-I solution, containing 1 mM MgSO_4 and 10.2 mM Na-Hepes at pH 7.4, before incubation with TdT enzyme. As a negative control, water was substituted instead of TdT enzyme.

Determination of RNA and Protein Synthesis

For determination of RNA synthesis rates, mixed cultures of neurons and confluent astrocytes as well as purified cultures of confluent astrocytes were prepared and plated in 24-well tissue culture trays as described above. Cells were washed once with 0.1 M PBS and pulse labeled with $0.5 \text{ ml}/\text{well}$ of media (DME/2% FCS) plus $1 \mu\text{Ci}/\text{ml}$ of $[5\text{-}^3\text{H}]\text{uridine}$ ($27 \text{ Ci}/\text{mmol}$) and $20 \mu\text{M}$ uridine for various time points up to 4 h, in a shaking metabolic incubator perfused with 5% CO_2 /95% air at 37°C . After the incubation period, the media was aspirated and the cells were washed twice with cold PBS. The cells were then lysed with cold 10% TCA and centrifuged at $12,000 \text{ g}$ for 15 min at 4°C . Aliquots of the supernatant were used for determination of the amount of free intracellular, radiolabeled uridine using a liquid scintillation counter. The pellet was solubilized with 1 M hyamine hydroxide (ICN Biochemicals) and aliquots used for determination of the amount of incorporated, acid-precipitable, radiolabeled uridine. RNA synthesis rates for neurons were determined by subtracting the amount of incorporation of $[^3\text{H}]\text{uridine}$ by astrocytes from that of mixed cultures and normalizing to cellular protein. To determine the effects of camptothecin, DRB and ActD on RNA synthesis, cells were incubated

with the inhibitors for 5 h as described above and the acid-precipitable counts under experimental conditions were divided by acid-precipitable counts under control conditions and normalized to protein levels as described previously (9). Protein synthesis was similarly determined in mixed cultures of neurons and astrocytes and confluent astrocyte cultures. Cells were incubated in leucine-deficient media and pulse labeled with $1 \mu\text{Ci}/\text{ml}$ of $[4,5\text{-}^3\text{H}]\text{L-leucine}$ ($77 \text{ Ci}/\text{mmol}$) and $20 \mu\text{M}$ L-leucine for 5 h in the presence or absence of increasing concentrations of CHX. Protein synthesis for neurons was determined by subtracting the amount of incorporated, acid-precipitable $[^3\text{H}]\text{leucine}$ by astrocytes from that of mixed cultures and normalizing to cellular protein.

Electronic Imaging

Bright-field phase, differential interference contrast, and fluorescence images were captured using a Dage CCD72 camera and Dage DSP2000 digital signal processor (capable of on-chip integration for low-light situations), and a Macintosh Quadra 700 with a Scion LG-3 frame grabber board. Images were analyzed with the NIH Image program (V 1.57, available by anonymous FTP from zippy.nimh.nih.gov) and further processed with Adobe Photoshop software. Final prints were produced using a Kodak 7700 color printer.

Time Lapse Videomicroscopy

Cortical neurons ($5.6 \times 10^4 \text{ cells}/\text{cm}^2$) were grown for 6–7 d in vitro on a preexisting monolayer of astrocytes in PLL-coated ($100 \mu\text{g}/\text{ml}$) 10 cm^2 Nunc chamber flasks. At the beginning of the observation period, cells were washed twice with PBS and incubated with DME/2% FCS with or without camptothecin. The flasks were sealed and placed on the stage of a

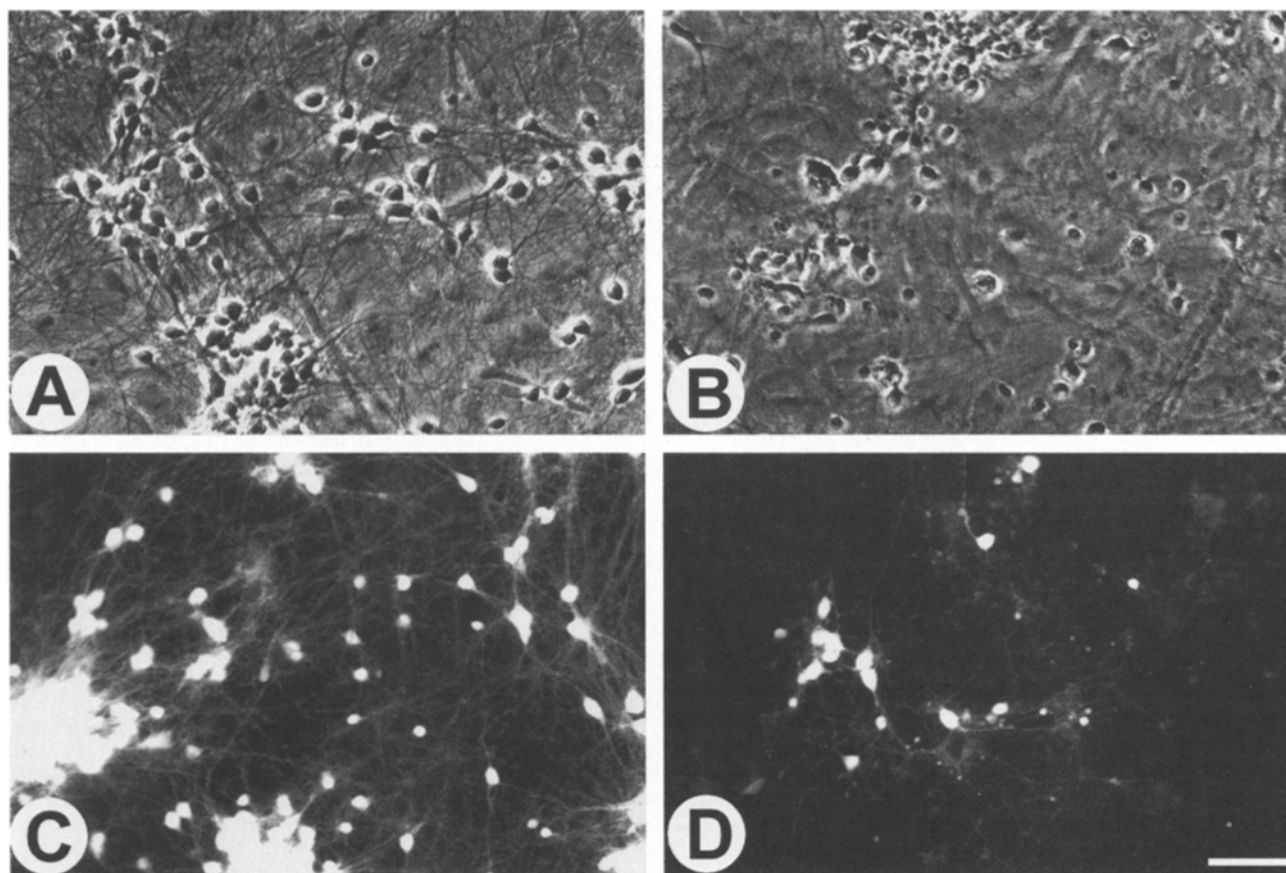


Figure 1. Camptothecin-induced cell death in mixed cultures of neurons and astrocytes. (A) Phase-contrast image of a control culture of dissociated embryonic rat cerebral cortical neurons at 6 d in vitro. (B) Phase-contrast image of culture after 24 h of treatment with $10 \mu\text{M}$ camptothecin. (C) Control culture labeled with CF demonstrating brightly stained neuronal somata and meshwork of neuritic processes. (D) Image of a culture treated with $10 \mu\text{M}$ camptothecin for 24 h and labeled with CF demonstrating fewer surviving neurons and extensive fragmentation of neurites. Bar, $50 \mu\text{m}$.

Nikon Diaphot-TMD inverted camera equipped with a stage box and Nikon NP2 thermostatic heater. Cultures were observed using a 40× phase contrast objective and images were recorded with an RCA TC-1005 camera and RCA TC-3700 time lapse video recorder at a time reduction of 2 min to 1 s (1:120). Selected frames were captured during video playback by the NIH Image Program using a Data Translation DT2255 frame grabber card.

Results

Camptothecin Induces Neuronal Cell Death

Camptothecin (10 μM) was added to mixed cultures of neurons and astrocytes as described in Materials and Methods, and survival was evaluated at 24 h. At this time, control cultures consisted of healthy, phase-bright neurons on top of the flat astrocyte monolayer (Fig. 1 A). In contrast, cultures treated with camptothecin contained fewer surviving neurons and showed accumulation of cell debris on the astrocyte monolayer (Fig. 1 B). Moreover, surviving neurons demonstrated neurite fragmentation and somal blebbing. In contrast, the astrocyte monolayer remained healthy and intact, with no apparent changes in astrocyte morphology. Incubation with CFDA provided a clearer demonstration of these morphological changes in neurons. In control cultures, neurons appeared healthy with an elaborate mesh of neurites and brightly stained somata (Fig. 1 C). In camptothecin-treated cultures, there was a reduction in CF fluorescence, due to both a decrease in the number of surviving neurons as well as a loss of neurites (Fig. 1 D). In addition, camptothecin-treated cultures contained a large amount of CF-stained debris, which is likely due to the hydrolysis of CFDA by neuronal apoptotic bodies.

We then determined the dose and time dependence of camptothecin-induced cell death. The dose dependence of toxicity was determined at 72 h. Mixed cultures of neurons and astrocytes were treated with camptothecin for 72 h, and neuronal survival was determined using CFDA assay.

Parallel cultures of confluent astrocytes were treated with camptothecin, and astrocyte survival was determined at 72 h using the MTT viability assay. Camptothecin treatment resulted in cell death with IC_{50} values of 0.44 and 6.9 μM for neurons and astrocytes, respectively (Fig. 2 A). The 16-fold difference in the IC_{50} values for camptothecin-induced toxicity of neurons and astrocytes demonstrated that astrocytes were more resistant, confirming the morphological observations.

The time course of cellular toxicity was similarly determined using both mixed cultures of neurons and astrocytes and purified cultures of astrocytes. Cultures were treated with 10 μM camptothecin, and neuronal and astrocyte survival was determined at 0, 24, 48, and 72 h. Neurons treated with camptothecin died more rapidly than astrocytes (Fig. 2 B). After 24 h, neuronal survival was $\sim 30\%$ of control values, whereas no astrocyte cell death was apparent. Astrocyte toxicity was not observed until 48 h, where survival was $\sim 83\%$ of control, untreated cultures. At 72 h, virtually all neurons had died, while $\sim 63\%$ of astrocytes were still viable.

Cell Death Is Independent of DNA Synthesis

The S-phase specificity of camptothecin has been supported by the fact that aphidicolin, an inhibitor of DNA polymerase α , prevented camptothecin-induced cell death in cycling cells (13, 28). To further confirm that DNA replication does not contribute to camptothecin-induced neuronal death, mixed cultures of neurons and astrocytes were treated with camptothecin in the presence and absence of increasing concentrations of aphidicolin, and neuronal survival was determined after 24 h *in vitro*. Aphidicolin was unable to protect neurons from camptothecin-induced neurotoxicity (Fig. 3), suggesting that DNA polymerase α activity does not contribute to camptothecin-induced neuronal death. In addition, aphidicolin treatment itself did not produce any significant degree of neuronal death.

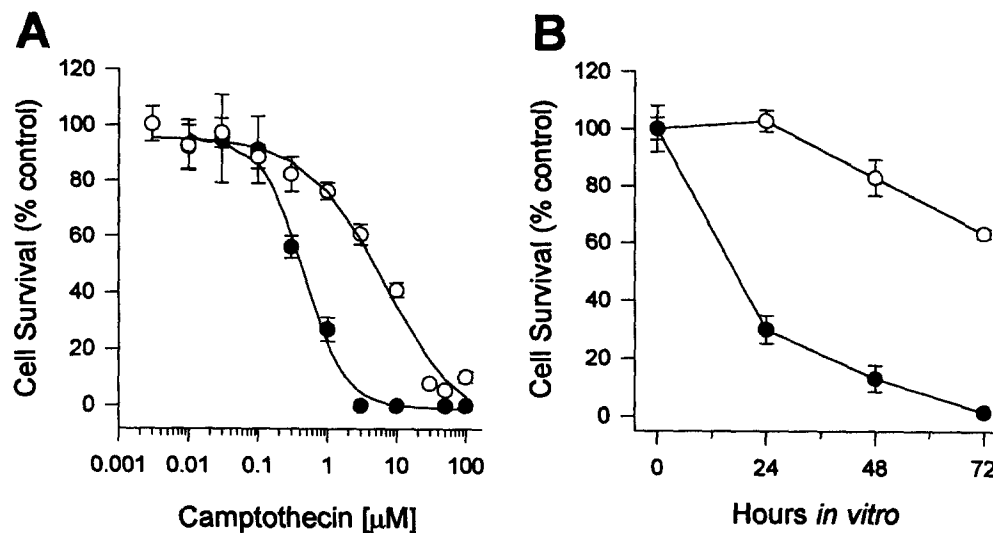


Figure 2. Dose-response and time course of camptothecin-induced cell death. (A) Dose-response for camptothecin-induced cell death. Mixed cultures were treated with various doses of camptothecin and cell survival was evaluated at 72 h after initiating treatment. Neuron and astrocyte survival were assayed by the CFDA and MTT assays, respectively. Data are expressed as percent control of the mean \pm SD ($n = 3-4$ per condition). The effect of camptothecin on both neuron and astrocyte survival was significant ($P < 0.0001$ by one-way ANOVA). (B) Time course of toxicity of

neurons and astrocytes evaluated after 10 μM camptothecin treatment. Neuron and astrocyte survival were assayed as described above. Data are expressed as percent control of the mean \pm SD ($n = 4$). The effects of camptothecin on neuron and astrocyte survival were significant ($P < 0.0001$ by one-way ANOVA). \circ , astrocytes; \bullet , neurons.

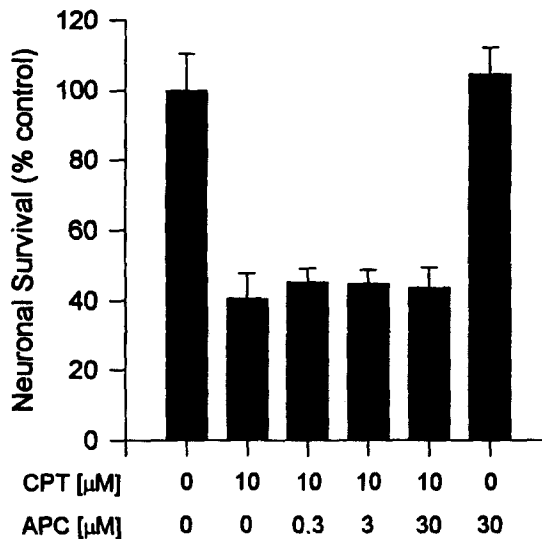


Figure 3. Aphidicolin does not prevent camptothecin-induced neuronal death. Mixed cultures were treated with 10 μM camptothecin in the presence of increasing concentrations of aphidicolin (APC) and neuronal survival was assayed at 24 h by the CFDA assay. Data is expressed as mean \pm SD ($n = 3-4$).

Camptothecin Treatment Induces Neuronal Apoptosis

Camptothecin treatment has been shown to induce apoptosis in proliferating cells (4, 54). Apoptosis is characterized by various morphological descriptions including condensation of nuclear chromatin, shrinking of the cytoplasm, and blebbing of the membrane with formation of apoptotic bodies. To determine if camptothecin-induced neuronal death was apoptotic or necrotic, mixed cultures of neurons and astrocytes were treated with camptothecin and analyzed by continuous time lapse video microscopy. In parallel, other cultures were treated with a high concentration of rotenone (1 μM), which inhibits complex-I of the mitochondrial electron transport chain and induces necrosis (25). In control cultures of 2% FCS alone, there was little change in neuronal survival at 24 h, although some neuronal migration was evident (Fig. 4 A). Rotenone treatment induced a rapid cell death within 3 h which morphologically consisted of cell swelling and disintegration characteristic of necrosis (Fig. 4 B). Camptothecin-induced neuronal death had a slower onset and was accompanied by a dramatic change in neuronal morphology (Fig. 4 C). Neuronal death was characterized by somal zeiosis (plasma membrane blebbing and contraction), fragmentation of neurites, and eventually cell body shrinkage leaving a phase-bright, cell "ghost." This morphology is characteristic of apoptosis and occurred over a period of 4-8 h. While most neurons died within 12-24 h, a few survived for longer times.

Apoptotic cell death is typically accompanied by condensation of nuclear chromatin (70). DNA-intercalating dyes can be used to visualize condensed DNA, which usually appears as several brightly-stained DNA "clumps" marginating at the nuclear envelope. To determine if camptothecin could induce apoptotic chromatin condensation in neurons, cells were treated with camptothecin (10 μM) and the nuclei were visualized by staining with the nuclear dye DAPI after 18 h of incubation. Under control

conditions, neuronal nuclei appeared smaller and more brightly stained than astrocytic nuclei (Fig. 5 A). In addition, the nuclei of both cell types appeared round and largely intact. After camptothecin treatment, many neurons had undergone nuclear chromatin condensation characteristic of apoptosis (Fig. 5 C, arrows), whereas astrocytes were unaffected (Fig. 5 C, arrowhead). After 18 h of camptothecin treatment, $24.2 \pm 9.6\%$ of the neurons had condensed nuclear chromatin, as compared with $1.4 \pm 0.1\%$ of the neurons in control cultures.

DNA fragmentation is considered the hallmark morphological feature of apoptosis and is characterized by the activation of a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease which cleaves DNA into 180-200 base pair oligonucleosomal size fragments which can be visualized by agarose gel electrophoresis (69). Neuronal apoptosis is typically accompanied by DNA fragmentation in vitro and in vivo (15). In addition, camptothecin treatment of cycling cells results in apoptotic DNA fragmentation (19). Therefore, we wished to determine if camptothecin could induce apoptotic DNA fragmentation in neurons. Evaluation of DNA fragmentation by agarose gel electrophoresis is impractical due to the presence of glial cells in our mixed cultures. Therefore, we utilized the TUNEL assay to specifically label and visualize those cells that contained nuclei with fragmented DNA. This assay takes advantage of the large numbers of free 3'-OH DNA ends generated by endonuclease cleavage, which can be labeled with digoxigenin-dUTP via the TdT enzyme. Camptothecin-induced cleavable-complex formation, itself, has been reported not to induce TUNEL labeling, presumably because topo-I is covalently linked to the free 3'-OH DNA end (24). Few labeled cells were observed in untreated control cultures (Fig. 6 A). However, cultures that were treated with 10 μM camptothecin for 18 h contained many labeled neurons (Fig. 6 B) characterized by darkly stained nuclei. Camptothecin treatment did not cause any labeling of astrocyte nuclei (Fig. 6 B). As a positive control, cultures were treated with DNase-I (1 $\mu\text{g}/\text{ml}$ for 20 min) before TdT labeling. In these cultures, both neuron and astrocyte nuclei labeled positively (data not shown). These data suggest that camptothecin treatment of neurons results in the activation of an endonuclease that induces apoptotic DNA fragmentation.

ATA Inhibits Camptothecin-induced Neuronal Death

Since camptothecin treatment induced apoptotic DNA fragmentation in neurons, we wished to determine whether ATA, an inhibitor of the putative apoptotic endonuclease (2), could prevent camptothecin-induced neuronal death. Neurons were therefore cotreated with ATA, up to 250 μM in the presence of 10 μM camptothecin for 24 h in vitro. ATA partially protected neurons from camptothecin-induced toxicity (Fig. 7). Treatment with ATA (250 μM) alone had no effect on neuronal survival. These results suggest that camptothecin treatment of neurons can result in the activation of an endonuclease which cleaves neuronal DNA during apoptosis.

Neuronal Death Is Mediated by DNA Topoisomerase-I

To determine if camptothecin-induced neuronal death is specific to agents which inhibit topo-I, stereoisomers of

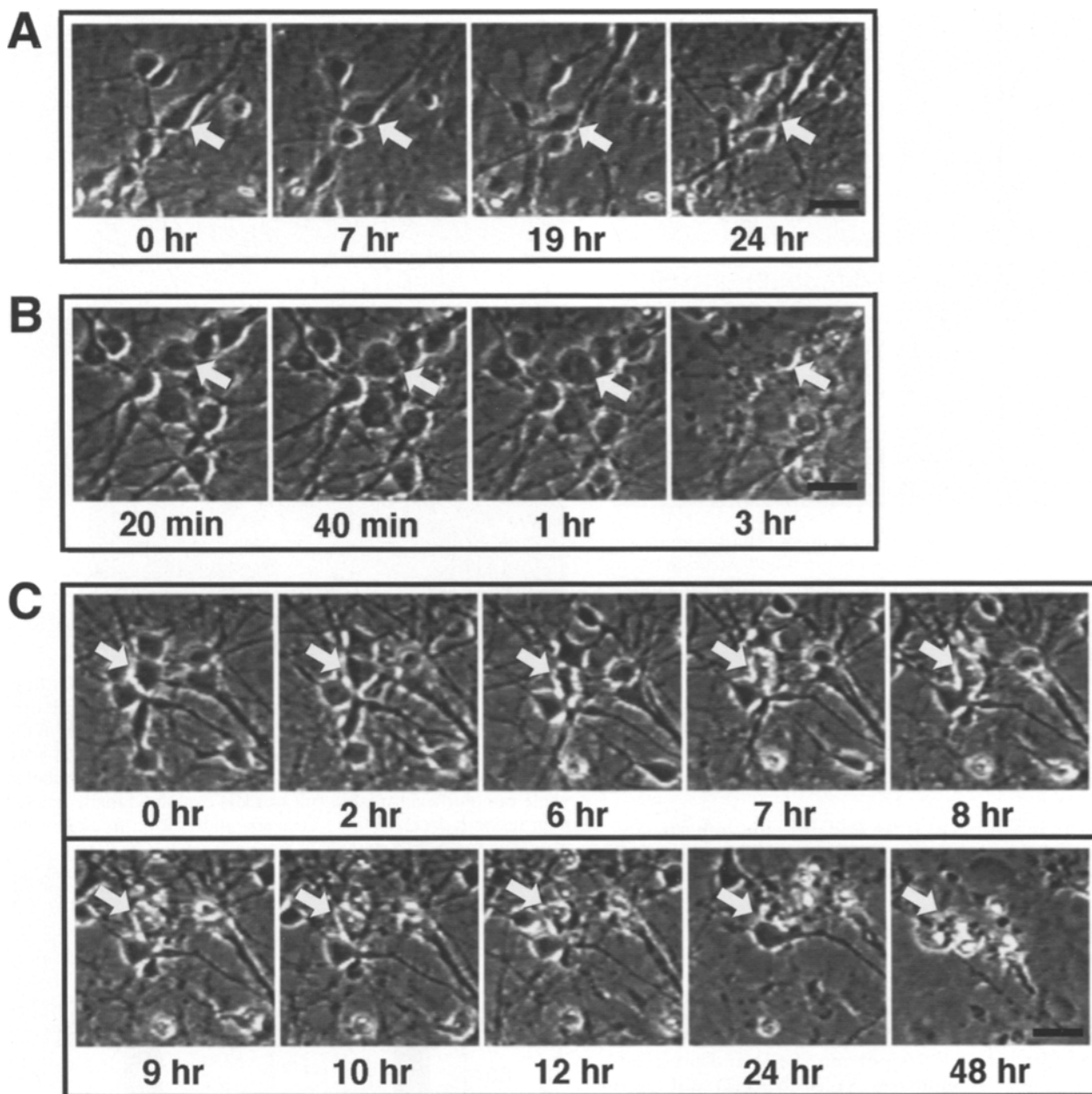


Figure 4. Camptothecin treatment of neurons results in morphological changes typical of apoptosis. Cortical neurons were grown for 6–7 d *in vitro* on a preexisting monolayer of astrocytes in slide chamber flasks. Cells were recorded by phase-contrast, continuous time-lapse videomicroscopy under (A) control conditions, (B) 1 μM rotenone treatment, or (C) 10 μM camptothecin. Little neuronal death was observed within 24 h in control conditions (see arrow, A), whereby rotenone induced a rapid cell death consisting of cell swelling and disintegration characteristic of necrosis (see arrow, B). Camptothecin-induced neuronal death was characterized by a slower onset associated with cytoplasmic blebbing and contraction that lasted \sim 4–8 h and was characteristic of apoptosis (see arrow, C).

the camptothecin analog, MDCPT, were used (Fig. 8). The 20(S)-isomer of MDCPT (S-MDCPT) is active while the 20(R)-isomer of MDCPT (R-MDCPT) is inactive in trapping topo-I on DNA and causing strand breaks (45). In addition, the stereospecificity of MDCPT correlates closely with its antitumor activity; S-MDCPT, which is more active than 20(S)-camptothecin, is one of the most potent known camptothecin analogues (50). Treatment of neurons with S-MDCPT resulted in a dose-dependent cell death. S-MDCPT was \sim 70 \times as potent as 20(S)-camptothecin in producing neural cell death (Fig. 8) over a 24-h

incubation period: the IC_{50} value for S-MDCPT was 0.081 μM as compared with an IC_{50} value for camptothecin of 5.5 μM . Treatment with the inactive isomer, R-MDCPT, did not cause neuronal cell death. These data strongly suggest that camptothecin-induced neuronal death is specifically mediated by inhibition of the target enzyme, topo-I.

RNA and Protein Synthesis Inhibitors Prevent Camptothecin-induced Neuronal Death

Neuronal apoptosis after trophic factor withdrawal can be

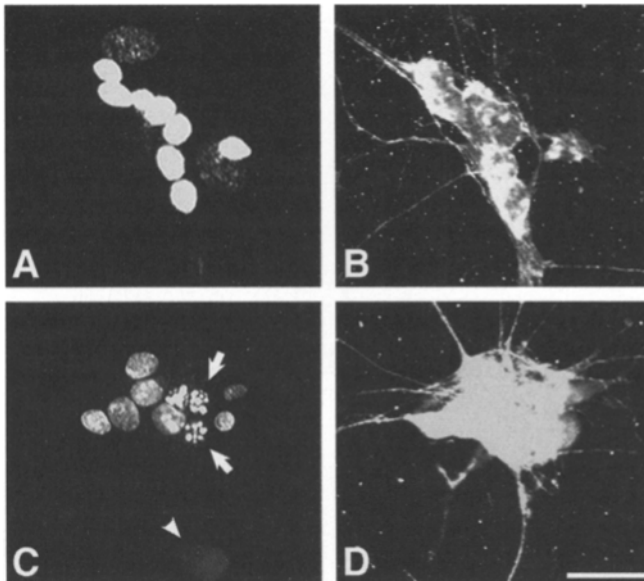


Figure 5. Camptothecin treatment of neurons results in apoptotic chromatin condensation. Cultures of neurons and astrocytes were treated without (*A* and *B*) or with (*C* and *D*) 10 μM camptothecin for 18 h *in vitro* and stained with DAPI (*A* and *C*) and neurofilament antibody (*B* and *D*). Camptothecin treatment resulted in the condensation of nuclear chromatin in neurons (*arrows, C*). The chromatin condensation pattern appeared as tight, compact clumps typically associated with cells undergoing apoptosis. There was no significant induction of chromatin condensation in astrocytes (*arrowhead, C*) by camptothecin treatment. Bar, 20 μm .

blocked with inhibitors of RNA and protein synthesis, suggesting that neuronal death depends upon the expression of a gene(s) involved in the apoptotic pathway (37). To determine whether camptothecin-induced neuronal death was also dependent upon active transcription or translation, ActD and DRB, inhibitors of RNA synthesis, were added to cultures along with 10 μM camptothecin. Neuron survival was assayed after 24 h. ActD can inhibit both rRNA and mRNA synthesis (48), while DRB is a specific, reversible inhibitor of RNA polymerase II and preferentially inhibits mRNA synthesis (8). Both ActD and DRB inhibit the death of sympathetic neurons after NGF deprivation (37). Neither ActD nor DRB were toxic when added alone at the maximum concentrations tested, suggesting that large-scale inhibition of transcription does not lead to neuronal cell death in our cultures. Both ActD and DRB protected neurons from camptothecin toxicity (Fig. 9). This protection was dose dependent; modest protection was observed at the lowest concentration of ActD used (0.005 $\mu\text{g}/\text{ml}$) while total protection was achieved at concentrations above 0.05 $\mu\text{g}/\text{ml}$ (Fig. 9 *A*). Significant protection from toxicity was only observed at the highest concentration of DRB used (100 μM), which offered almost complete inhibition of camptothecin-induced cell death (Fig. 9 *B*). The IC_{50} values for both ActD and DRB correlate with their ability to effectively inhibit RNA synthesis (48), suggesting that transcription is necessary for camptothecin-induced neuronal apoptosis. Since camptothecin has also been reported to inhibit RNA synthesis, we evaluated the ability of 10 μM camptothecin, 100 μM DRB, and

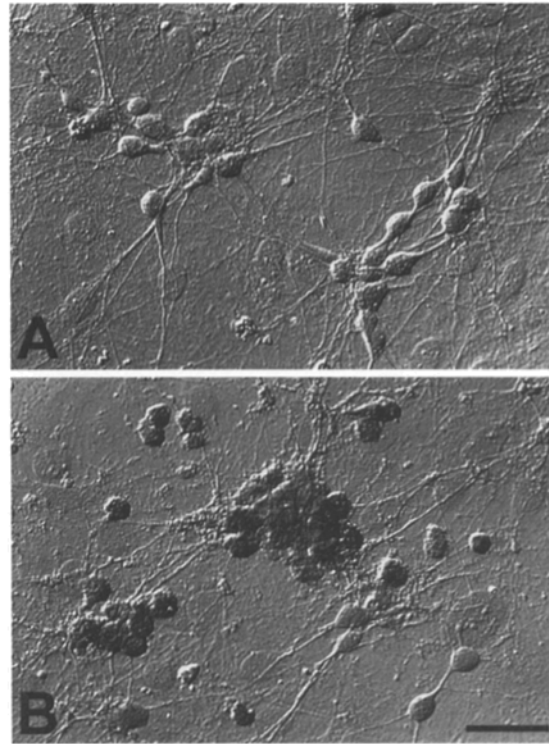


Figure 6. Camptothecin treatment induces apoptotic DNA fragmentation in neurons. Mixed cultures were treated with and without 10 μM camptothecin for 18 h. Cultures were labeled with the TUNEL assay and differential interference contrast images were captured. Untreated cultures (*A*) were not significantly labeled while camptothecin-treated cultures (*B*) contained many labeled neurons with darkly stained nuclei. Camptothecin treatment did not result in positive staining of astrocytes (*B*). Bar, 30 μm .

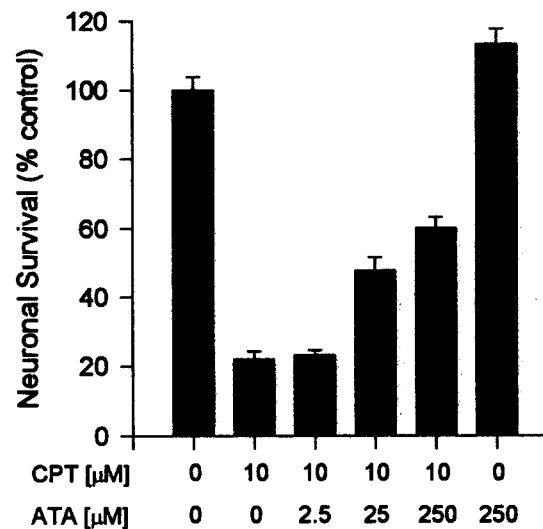


Figure 7. ATA inhibits camptothecin-induced neuronal death. Cultures were incubated with 10 μM camptothecin in the presence of increasing concentrations of ATA. Neuronal survival was determined at 24 h by the CFDA assay. Data is expressed as mean \pm SD ($n = 3-4$). ATA significantly prevented neuronal death after camptothecin treatment ($P < 0.0001$ by one-way ANOVA).

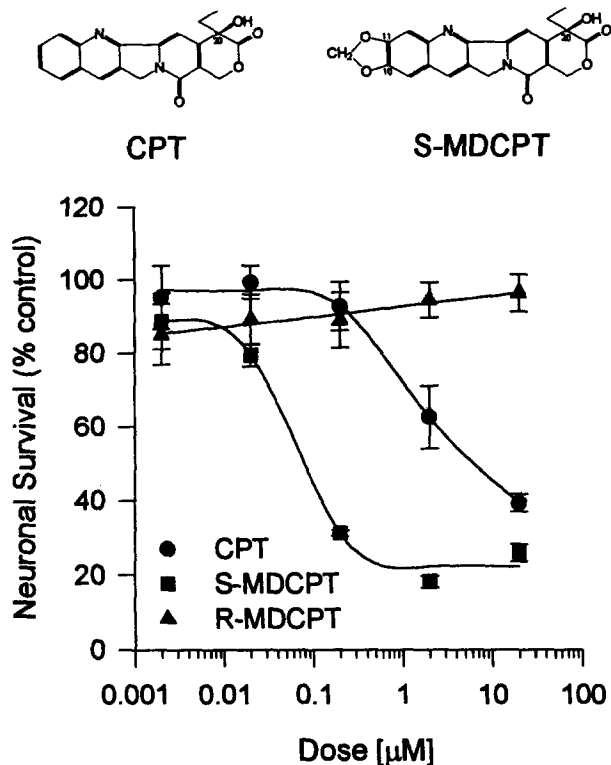


Figure 8. Camptothecin-induced neuronal death is mediated by DNA topoisomerase-I. Dose-response curve of neuronal cell death evaluated after treatment with camptothecin or stereoisomers of the camptothecin analog, MDCPT. Neurons were treated with the above indicated agents and survival was evaluated by the CFDA assay at 24 h in vitro. Data are expressed as percent control of the mean \pm SD ($n = 3-4$). The effects of camptothecin and S-MDCPT on neuronal survival were significant ($P < 0.0001$ by one-way ANOVA), while the effects of R-MDCPT were not significant. The chemical structures for both 20(S)-camptothecin (CPT) and S-MDCPT are depicted.

0.5 $\mu\text{g/ml}$ ActD to inhibit transcription in astrocyte and mixed cultures. After 5 h of incubation, these concentrations of DRB and ActD inhibited [^3H]uridine incorporation by 41 and 90%, respectively, while camptothecin in-

hibited [^3H]uridine incorporation by 39% (data not shown). Thus, the toxicity of camptothecin cannot be ascribed to an inhibition of RNA synthesis.

To investigate if camptothecin-induced cell death is dependent upon new protein synthesis, neurons were co-treated with the protein synthesis inhibitors cycloheximide (CHX) and anisomycin (ANM). Neither of these inhibitors, when used alone at the maximal concentration, were neurotoxic within the 24-h treatment period. CHX was used in a range of concentrations from 5 ng/ml to 0.5 $\mu\text{g/ml}$, since similar concentrations of CHX have been shown to inhibit protein synthesis in sympathetic neurons (37). In addition, we found that 0.5 $\mu\text{g/ml}$ CHX inhibited cortical neuronal protein synthesis by $\sim 50\%$ after a 5-h treatment period; maximal inhibition of protein synthesis was achieved at 1 $\mu\text{g/ml}$ (data not shown). Significant protection by CHX was only observed at the highest concentration used, 0.5 $\mu\text{g/ml}$ (Fig. 10 A). Similarly, addition of ANM prevented neuronal death by camptothecin (Fig. 10 B); protection was observed at the lowest concentration used, 0.5 μM . The surviving neurons in cultures cotreated with camptothecin and protein synthesis inhibitors appeared healthy and identical to neurons in control conditions and after treatment with CHX and ANM alone (data not shown). These data suggest that protein synthesis is necessary for the induction of neuronal death by camptothecin. This also demonstrates that camptothecin is probably not toxic to neurons by its ability to inhibit transcription, and thereby indirectly affecting protein synthesis.

Camptothecin Sensitivity Is Correlated with Levels of Transcription and Topo-I Expression

Topo-I is involved in both DNA replication and transcription. In other systems, the sensitivity to camptothecin has been correlated with levels of topo-I protein expression (12). We therefore evaluated the levels of topo-I in neurons and astrocytes. Protein was isolated from E 17 rat cerebral cortex as well as from cortical neurons after 2 d in culture and confluent cultures of purified cerebral cortical astrocytes. The extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a polyclonal antibody to human topo-I which also recog-

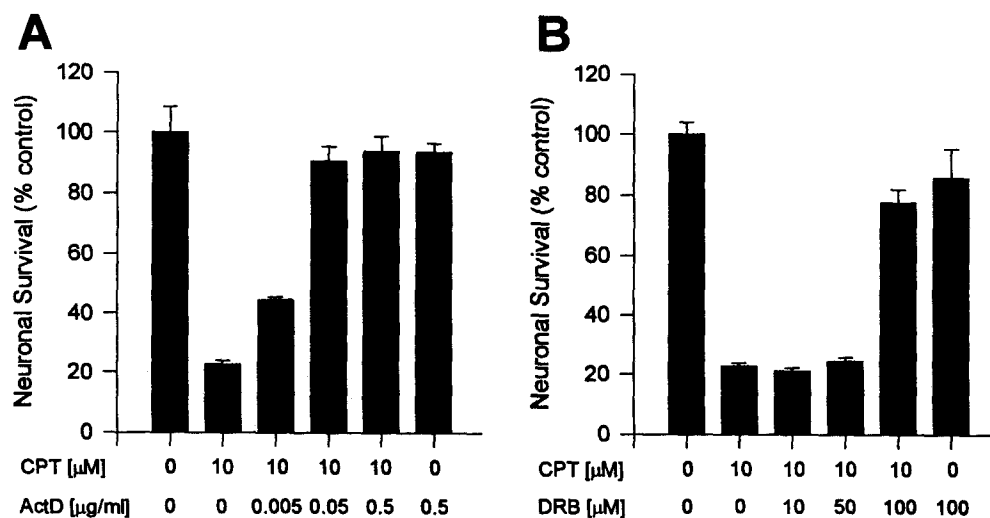


Figure 9. RNA synthesis inhibitors prevent camptothecin-induced neuronal death. Mixed cultures were treated with 10 μM camptothecin with increasing concentrations of the RNA synthesis inhibitors, (A) ActD or (B) DRB. Neuronal survival was evaluated at 24 h with the CFDA assay. Data are expressed as mean \pm SD ($n = 3-4$). The effects of ActD and DRB on neuronal survival were significant ($P < 0.0001$ by one-way ANOVA).

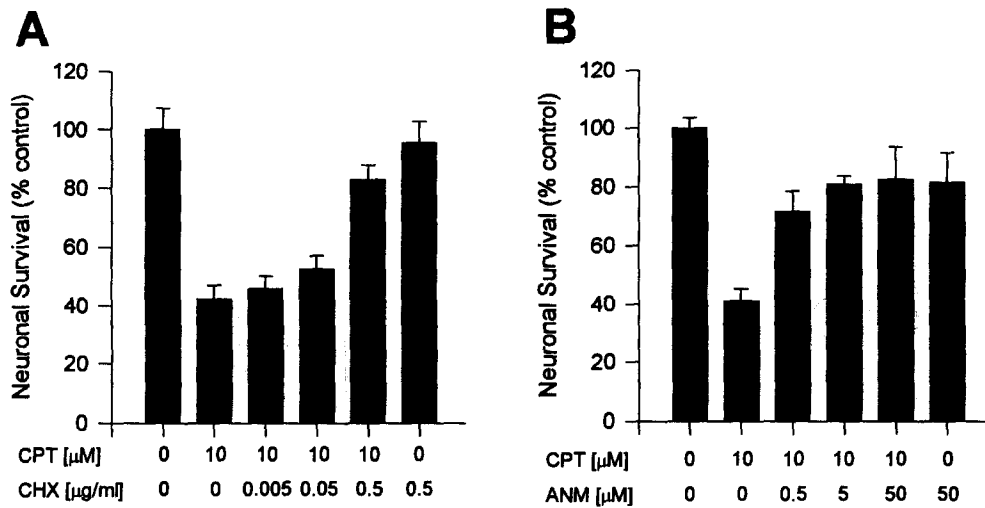


Figure 10. Protein synthesis inhibitors prevent camptothecin-induced neuronal death. Mixed cultures were treated with 10 μ M camptothecin with increasing concentrations of the protein synthesis inhibitors, (A) CHX or (B) ANM. Neuronal survival was evaluated at 24 h with the CFDA assay. Data are expressed as mean \pm SD ($n = 3-4$). The effects of CHX and ANM on neuronal survival were significant ($P < 0.0001$ by one-way ANOVA).

nizes the rat 100-kD topo-I. The immunoblots demonstrate that topo-I levels are significantly higher in embryonic brain and cultures of neurons than in the astrocyte cultures, although astrocytes did express a small amount of topo-I (Fig. 11). When the pixel intensity of the bands were analyzed, cultured neurons and neurons from embryonic brain expressed ~ 2.3 - and 4.4-fold higher topo-I levels than astrocytes, respectively. These data suggest that neurons might be more sensitive to camptothecin compared to astrocytes due to differences in topo-I expression.

Given that the degree of cell division in our astrocyte cultures is low, the primary function of topo-I would be most likely to relieve stress in DNA generated by transcription. We therefore evaluated levels of transcriptional activity of neurons in mixed cultures as compared with the level in purified astrocyte cultures by measuring [3 H]uridine incorporation over time. Neurons incorporated $\sim 5.5 \times$ the amount of [3 H]uridine on a per unit protein basis as compared with astrocytes (data not shown). Taken together with the data on the prevention of cell death by transcriptional inhibition, the differences in sensitivity of neurons and astrocytes to camptothecin appears to be related to their levels of transcription and topo-I expression.

Discussion

Our data demonstrate that camptothecin, a cell cycle-specific anticancer agent, exhibited significant toxicity toward postmitotic central nervous system neurons in culture. Camptothecin-induced neuronal death was accompanied by DNA fragmentation and chromatin condensation, cell blebbing, cytoplasmic shrinkage, and fragmentation of neurites, all characteristics of neuronal apoptotic cell death. Furthermore, neuronal death was inhibited by drugs which prevent RNA and protein synthesis, suggesting that the camptothecin-induced death of neurons was dependent upon active transcription and translation. Astrocytes were less sensitive to camptothecin, even though some of the astrocytes in these cultures were proliferating. Astrocytes expressed less topo-I protein compared with cultured neurons and embryonic brain suggesting that the level of topo-I expression might be an important determi-

nant in the sensitivity of neural cells to topo-I inhibitors. In addition, astrocytes incorporated less [3 H]uridine than neurons, suggesting that the degree of RNA synthesis might also contribute to camptothecin sensitivity. With the use of stereospecific topo-I inhibitors, we demonstrated that neuronal death is a direct result of inhibition of topo-I, suggesting that the function of this enzyme is essential for neuronal survival.

Camptothecin Causes Neuronal Apoptosis

Camptothecin induces an apoptotic death in many different cell types, and our data clearly demonstrate that it does so in nondividing neurons. Within 24 h after camptothecin treatment, neurons undergo the same apoptotic morphological changes which occur after trophic factor deprivation (15): the plasma membrane begins to undergo dramatic blebbing and contraction (zeiosis) and the neurites begin to disintegrate. This is followed by cytoplasmic shrinkage, which eventually leaves a small, round, phase-bright, cell ghost. In addition, camptothecin-treated neurons undergo chromatin condensation and DNA fragmentation. At the highest concentrations used, inhibitors of RNA and protein synthesis fully protect against camptothecin toxicity. Protection by these agents is typically observed in neuronal apoptosis (37). Apoptosis was also re-

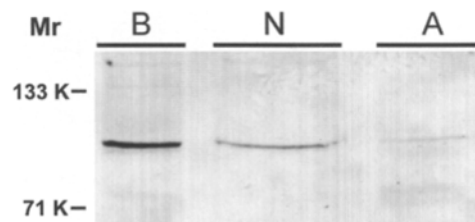


Figure 11. Immunoblot of topo-I protein in neural tissues. Extracts of E 17 rat cerebral cortex (B), E 17 neurons after 2 days in culture (N), and purified cultures of newborn rat cerebral cortical astrocytes (A) were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose. Topo-I immunoreactivity to an anti-human rabbit polyclonal antibody was visualized as a single 100-kD band.

duced by ATA, an endonuclease inhibitor. Although ATA normally fully protects many neurons from apoptosis (2), it offered only partial protection against camptothecin-induced cell death. It is possible that more than one endonuclease which is less sensitive to inhibition by ATA, might be active during the apoptotic process. In addition, our studies were performed with rat cerebral cortical neurons, which may differ from other neurons in their sensitivity to endonuclease inhibition. Taken together, these data demonstrate that the topo-I inhibitor, camptothecin, can induce apoptosis in neurons.

Camptothecin Toxicity Is Mediated by Topoisomerase-I

Topo-I is the only known target of camptothecin (21). The involvement of topo-I in camptothecin-induced neurotoxicity was established by the use of stereospecific isomers of the camptothecin analogue, MDCPT. Topo-I is an enzyme used in the relaxation of DNA supercoiling during replication and transcription (for review see 65). Topo-I catalyzes the “breakage-reunion” reaction of DNA, resulting in the separation and rejoining of one DNA strand. This activity allows the DNA to “swivel” in response to a torsional stress generated by the movement of the replication and/or transcription machinery along the DNA double-helix. Camptothecin does not bind to topo-I or DNA alone but binds to a topo-I/DNA complex and traps topo-I in an intermediate state, thereby effectively inhibiting the rejoining step of the breakage-reunion reaction. This results in the formation of a drug/topo-I/DNA complex (the cleavable complex) (27) with topo-I covalently attached to the 3'-OH end of the severed DNA backbone, thus creating a DNA single-strand break. It is the collision of the DNA replicational machinery with the cleavable-complex that is thought to generate potentially lethal DNA double-strand breaks (28). This is supported by the fact that aphidicolin, an inhibitor of replicative DNA polymerase α , abolishes camptothecin-induced toxicity in proliferating cells (28).

In our experiments, aphidicolin offered no protection against camptothecin toxicity, a not unexpected result, since quiescent neurons do not express DNA polymerase α (66). Thus, there must be alternative mechanisms, independent of DNA synthesis, by which inhibition of topo-I can produce cell death. We propose that DNA damage occurs by the collision of the transcriptional machinery with the cleavable-complex, an event analogous to camptothecin-mediated, replicationally driven DNA strand breaks. In vitro transcription assays demonstrate that the transcriptional machinery can indeed collide with the cleavable-complex, on the DNA template strand, resulting in the premature termination of transcription (3). Moreover, topo-I is preferentially found at transcriptionally active regions of DNA (65) and can be found in significant quantities in the nucleolus (41), the primary site for transcription of rRNA in the cell. Treatment of cells with high concentrations of camptothecin results in transcriptional inhibition of mainly large rRNA and mRNA transcripts (31, 71). While some transcriptional inhibition by camptothecin takes place in our experiments, this is unlikely to contribute to toxicity, since the other transcriptional inhibitors, both more effective at inhibition of RNA synthesis, were without toxicity when used by themselves. In addition, if

camptothecin-induced neuronal death were due to general transcriptional inhibition, cotreatment with ActD or DRB should result in additive toxicity. Our data, which demonstrate that camptothecin neurotoxicity was reduced by either ActD or DRB, suggest that cell death could be due to DNA damage caused during the transcriptional process.

Sensitivity to camptothecin could also be due to the lack of efficacy of DNA repair. The repair of camptothecin-induced damage to cells depends upon functional repair mechanisms (26). Yeast cells with a deletion of the *RAD52* DNA repair gene are hypersensitive to camptothecin and cannot repair DNA double-strand breaks formed after camptothecin treatment (44). Moreover, cell lines that are deficient in the DNA repair enzyme poly(ADP-ribose) polymerase are hypersensitive to camptothecin (6), as are cells derived from individuals with the inherited disease Cockayne Syndrome (CS) (55), a defect in transcription-coupled DNA repair. CS cells are also hypersensitive to UV and ionizing radiation-induced DNA damage (34, 61). Interestingly, patients with CS are typically born with severe neurological abnormalities characterized by massive neuronal and glial cell death, demyelination, and the presence of Alzheimer's-like neurofibrillary tangles (53). Therefore, neurons may be relatively deficient in DNA repair and consequently sensitive to DNA damage produced by topo-I inhibitors.

Astrocytes Are Less Sensitive to Camptothecin

Astrocytes were consistently less sensitive than neurons to toxic actions of camptothecin. This seems counterintuitive, since astrocytes are not terminally differentiated and proliferate at a low rate in our cultures. Differences in rates of transcription might explain this difference in sensitivity to camptothecin. Our data indicate that the rate of incorporation of [³H]uridine was ~5.5 times greater into cultured rat cortical neurons than astrocytes, consistent with previous data showing that neurons have two to three times the transcriptional activity of glial cells (22). In addition, use of cell-free transcriptional assays demonstrated that neurons also have double the number of available RNA transcriptional initiation sites on chromatin compared to glial cells (52). The fact that neurons had significantly higher levels of topo-I than astrocytes might also explain the greater sensitivity of neurons to camptothecin. Overexpression of topo-I in yeast (21) and BHK cells (36) leads to camptothecin hypersensitivity, suggesting that the neurons are more sensitive to camptothecin because they express higher levels of topo-I. Another possibility is that there may be differences in topo-I activity via posttranslational modification. Topo-I activity can be modulated by both phosphorylation and ADP ribosylation of the enzyme (29). However, the mechanisms by which topo-I activity is regulated in neurons and astrocytes are not known.

Another explanation for the different camptothecin sensitivities might be that astrocytes differ from neurons in their apoptotic pathway. Astrocytes are particularly resistant to other conditions which produce apoptosis in neurons, such as ionizing radiation (8a), oxidative stress (20), and staurosporine treatment (32), suggesting that important differences between these cell types might be in the apoptotic machinery itself. For example, expression of the

apoptosis-inhibiting proto-oncogene, *bcl-2*, is higher in cultured mouse astrocytes than neurons (38), and constitutive expression of *bcl-2* reduces camptothecin-induced apoptosis in cycling cells (64a). Therefore, neurons and astrocytes may express different inducers and suppressors of apoptosis which ultimately modulate topo-I-mediated cell death. Finally, astrocytes repair DNA damage from ionizing radiation faster than neurons (8a), suggesting that differences in DNA repair might mediate camptothecin sensitivity in these cell types. Understanding why these cells differ in their sensitivity to camptothecin could have broad implications for the understanding of apoptosis related to cancer and neuronal development as well as topo-I function in the CNS.

Summary

Our results demonstrate that camptothecin is toxic to ter-

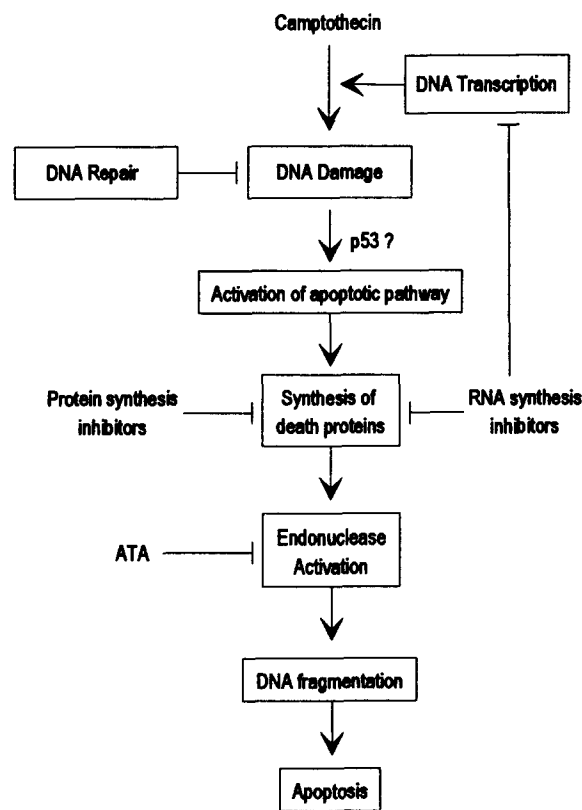


Figure 12. Proposed model for camptothecin-induced neuronal apoptosis. Camptothecin binds and inactivates topo-I forming the cleavable-complex. Collision of the transcription machinery with the cleavable-complex causes DNA damage, which leads to apoptosis if not repaired. The rate and competency of neuronal DNA repair would serve ultimately to determine the sensitivity of neurons to camptothecin. If unrepaired, DNA damage is then recognized by proteins such as the tumor suppressor *p53*, which then activate the apoptotic pathway and initiate the synthesis of proteins which cause cell death. Under this model, inhibition of transcription can block either DNA damage or expression of proteins involved in apoptosis. Protein synthesis inhibitors act similarly by blocking the expression of apoptotic factors. Finally, expression or activity of an apoptotic endonuclease(s) is increased, which causes neuronal DNA fragmentation and can be inhibited by ATA.

minally differentiated cells that do not progress through S-phase. Although the majority of evidence supports a role for camptothecin toxicity mediated in the S-phase, accounts of non-S-phase killing by camptothecin have been reported (5, 11). In addition, treatment of a variety of human cell lines with camptothecin resulted in cell death which did not correlate with cell cycle kinetics (47). Our data suggest that toxicity to nondividing cells is correlated with the level of active RNA transcription. We propose a model to illustrate the process of camptothecin-induced apoptotic death in postmitotic neurons (Fig. 12). In this model, the initial process is the binding of camptothecin to topoisomerase-I and DNA to form the cleavable-complex; this inactivation of topo-I then results in the formation of DNA strand breaks during the transcriptional process. While the signal transduction pathways involved have not yet been elucidated, we posit a role for tumor suppressor protein *p53* in initiating the apoptotic process. Camptothecin induces *p53* expression (58) and *p53* is required for apoptosis following DNA damage by radiation or anticancer agents (35); *p53* expression in neurons is increased after DNA damage (68). Downstream events would then include the synthesis of proteins involved in the apoptotic process, which can be inhibited by inhibitors of either RNA or protein synthesis. Finally, the cleavage of DNA is through the activation of an apoptotic endonuclease, the activity of which is inhibited by ATA. The major feature of this model is that DNA damage to neurons is during the transcriptional process. However, since inhibition of transcription would be expected to inhibit both DNA damage and the synthesis of genes involved in the apoptotic program, additional experiments to address the signal transduction pathways in camptothecin-mediated apoptosis will be necessary. Since these signal transduction pathways involve genes which also participate in the regulation of the cell cycle, the use of terminally differentiated neurons for these studies will permit the detailed molecular analysis of the apoptotic events uncluttered by the presence of a functioning cell cycle.

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