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# Induction of DNA Damage, Inhibition of DNA Synthesis, and Suppression of c-*myc* Expression by the Topoisomerase I Inhibitor, Camptothecin, in MCF-7 Human Breast Tumor Cells

*Pramod T. Jain, Frank A. Fornari, Joyce K. Randolph, Michael S. Orr and David A. Gewirtz**

Departments of Pharmacology and Toxicology, Virginia Commonwealth University, Medical College of Virginia, Richmond, VA 23298, U.S.A.

**ABSTRACT.** Previous work from this laboratory has demonstrated an association between the suppression of c-*myc* expression and the antiproliferative activity of both topoisomerase II inhibitors and ionizing radiation in MCF-7 breast tumor cells. These findings suggested that suppression of c-*myc* expression could be related to the induction of DNA damage in this cell line. The present studies were designed to determine whether the inhibition of topoisomerase I (and the consequent induction of DNA strand breaks) would also result in the suppression of c-*myc* expression. At camptothecin concentrations of 1 μM and below, there was no detectable damage (single- or double-strand breaks) in bulk DNA or suppression of c-*myc* expression. At camptothecin concentrations of 5, 10, and 25 μM, where suppression of c-*myc* expression was observed, strand breaks in bulk DNA were also detected. These findings are consistent with the idea that suppression of c-*myc* expression could be a component of the DNA damage response pathway in MCF-7 breast tumor cells. In contrast to the absence of detectable damage to bulk DNA or suppression of c-*myc* expression at the lower concentrations of camptothecin, DNA synthesis was inhibited over the entire range of drug concentrations and demonstrated a strong correspondence with growth inhibition. These observations support the concept that growth inhibition of MCF-7 cells by camptothecin is closely related to the early suppression of DNA synthesis. BIOCHEM PHARMACOL **55**;8:1263–1269, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** MCF-7 breast tumor; camptothecin; c-*myc*; DNA strand breaks; DNA synthesis

The topoisomerases are a family of enzymes that are essential for a variety of DNA-associated processes such as replication, transcription, and recombination [1, 2]. The two primary eukaryotic DNA topoisomerases function in similar but distinct fashions to modify DNA conformation. Topoisomerase I catalyzes DNA relaxation via a transient single-strand break, with covalent linking of the enzyme to the 3′-phosphotyrosyl end of the broken DNA strand. Topoisomerase II catalyzes topological crossing of two double-strand DNA segments via a transient double-strand break; each subunit of the homodimeric protein forms a covalent bond with the 5′-phosphoryl end of the broken DNA strands. Interference with DNA religation by the topoisomerase inhibitors results in the production of transient DNA strand breaks and DNA–protein complexes that appear to be critical for the cytostatic/cytotoxic effects of these drugs [2]. Camptothecin, an antitumor drug, is thought to act by inhibiting the religation capacity of topoisomerase I [3–6], resulting in the production of a drug-stabilized topoisomerase I–DNA complex [7]. Furthermore, cell killing may be a consequence of arrest of replication fork progression by this complex [7–9] and the induction of double-strand breaks in nascent DNA [10].

The nature of the signaling events leading to growth arrest and/or cell death subsequent to the perturbation of DNA function (formation of the DNA–topoisomerase I complex, replication fork arrest, and DNA strand breakage) have not been fully resolved. Previous work from this laboratory has demonstrated that topoisomerase II inhibitors such as VM-26,† *m*-AMSA, and doxorubicin as well as ionizing radiation suppress the expression of c-*myc* [11–14]; this early suppression of c-*myc* expression was determined to be predictive of the extent of growth inhibition [11–14], suggesting that c-*myc* may be closely linked with the signal transduction pathway, leading to growth arrest in this tumor cell line. We have reported further that growth

* Corresponding author: Dr. David A. Gewirtz, Department of Medicine, Medical College of Virginia, P.O. Box 980230, Richmond, VA 23298. Tel. (804) 828-9523; FAX (804) 828-8079: E-mail: gewirtz@gems.vcu.edu.

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† *Abbreviations:* VM-26 (teniposide) 4′-demethylepipodophyllotoxin-4-(4,6-*O*-thenylidene-β-D-glucopyranoside); *m*-AMSA, 4′-(9-acridinylamino) methanesulfon-*m*-anisidide; GAPDH, glyceraldehyde phosphate dehydrogenase; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

inhibition by VM-26, *m*-AMSA, and doxorubicin closely parallels the inhibition of DNA synthesis in MCF-7 breast tumor cells [11–13].

The studies in this report were designed to determine whether the antiproliferative activity of the topoisomerase I inhibitor camptothecin was similar to that of the topoisomerase II inhibitors in terms of its effects on DNA synthesis and the suppression of c-*myc* expression. Whereas drug effects on DNA synthesis demonstrated a close correspondence with growth inhibition over a broad range of camptothecin concentrations (0.1–25 μM), growth inhibition occurred in the absence of the suppression of c-*myc* expression at the lower range of drug concentrations (0.1–1 μM). These findings suggest that inhibition of DNA synthesis is a common early event that is predictive for growth inhibition by both topoisomerase I and topoisomerase II inhibitors in the MCF-7 breast tumor cell line. Suppression of c-*myc* expression occurs only at those concentrations of camptothecin where bulk DNA damage is evident, consistent with the concept that alterations in c-*myc* expression may represent a component of the DNA damage response pathway in MCF-7 breast tumor cells.

## MATERIALS AND METHODS

### *Materials*

Dulbecco's Modified Eagle's medium (DMEM, 56-439) was obtained from Hazelton Research Products; L-glutamine, penicillin/streptomycin (10,000 units of penicillin/mL and 10 mg of streptomycin/mL), and fetal bovine serum were obtained from Whittaker Bioproducts; defined bovine calf serum was obtained from Hyclone Laboratories. Trypsin-EDTA (10x; 0.5% trypsin, 5.3 mM of EDTA) was obtained from Life Technologies, and maintained as a frozen stock. Camptothecin was obtained from the Sigma Chemical Co., dissolved in DMSO (Aldrich Biochemicals), and maintained as a frozen stock solution for a maximum period of 3 weeks. Drug was diluted in incubation medium on the day of the experiment. The radiolabeled compound [$^3$H]thymidine (75 Ci/mmol) was obtained from ICN Radiochemicals. [α-$^{32}$P]dCTP (3000 Ci/mmol) was obtained from DuPont/NEN Research Products. Thymidine and trichloroacetic acid were obtained from Sigma. The restriction endonucleases were obtained from New England Biolabs, and the nick-translation kit was obtained from Life Technologies.

### *Probes and Constructs*

The c-*myc* probe, an *Eco*RI/*Cla*I fragment of PMC41 3RC containing the third exon of the human c-*myc* gene [15], was obtained from Dr. Eric Westin of the Medical College of Virginia. The GAPDH probe is a 780 bp *Pst*I/*Xba*I cDNA fragment from a pBR322 vector. This fragment was cloned from human fetal liver and binds to the sequence translated into the first 250 amino acids. The GAPDH cDNA was obtained from the American Type Culture Collection.

### *Cell Line*

The MCF-7 breast tumor cell line was provided by the laboratory of Dr. Kenneth Cowan at the National Cancer Institute. Cells were maintained as monolayers in DMEM supplemented with glutamine (0.292 mg/mL), penicillin/streptomycin (0.5 mL/100 mL of medium), 5% fetal bovine serum, and 5% defined bovine serum. All cells were cultured at 37° in an atmosphere of 5% $CO_2$ and 100% humidity. For the studies described in this manuscript, cells were subcultured at densities where the cells were maintained in logarithmic growth during the assay procedure.

### *Growth Inhibition using the MTT Assay*

The capacity of camptothecin to interfere with growth of the MCF-7 breast tumor cell line was determined using the MTT dye assay [16, 17], as described in detail previously [18]. Briefly, cells subcultured at a density of $1 \times 10^4$ cells/mL in 96-well microplates (Costar) were incubated with camptothecin for 3 hr at 37°, washed free of drug, and incubated in fresh medium for an additional 72 hr. Medium was removed by gentle inversion of the microplate, and the cells were washed with PBS prior to incubation with 100 μL of MTT solution (2 mg/mL of MTT in PBS; filtered as needed). MTT is converted to a blue formazan product by mitochondrial succinate dehydrogenase. This product was eluted from cells by the addition of 100 μL of DMSO, and absorbance at 540 nm was determined using an EL310 EIA autoreader (Biotek Instruments).

### *Alkaline Unwinding (for Detection of Combined Single- and Double-Strand Breaks)*

Bulk (single-strand) damage to DNA was determined using the alkaline unwinding procedure [19] as described in detail previously [11]. Cells in 75-cm$^2$ flasks were exposed to camptothecin or vehicle control for 2 hr at 37°. Following the drug exposure, the cells were washed with ice-cold PBS on ice (pH 7.4) and released from the flasks by an incubation with 0.05 mg/mL of trypsin in 0.02 mg/mL of EDTA for 5 min at 37°; cells were collected in ice-cold PBS (pH 7.4) and centrifuged at 4°. Following centrifugation, the cell pellet was resuspended in ice-cold PBS, and the cellular number was determined prior to analysis for strand breakage. This assay is based on the differential binding and fluorescence of bisbenzimide trihydrochloride (Hoechst 33258) to single- and double-strand DNA after a fixed period of alkaline denaturation. Each condition ($6 \times 10^6$ cells/condition) was subdivided into three different groups: (a) double-strand DNA control, with no alkali-induced DNA unwinding; (b) cells treated for a 10-min alkaline unwinding period; and (c) total single-strand DNA, where cells were sonicated before alkaline unwinding. F-values,

defined as: F = (alkali-treated DNA − single-strand DNA)/(double-strand DNA control − single-strand DNA) were determined in quadruplicate, and then converted to rad equivalence based on the standardization of DNA damage using a $^{137}$cesium irradiator to produce dose-dependent quantities of strand damage.

### *Neutral Elution Assay for the Induction of Double-Strand Breaks in DNA*

Strand breaks in DNA were analyzed further using the neutral elution assay procedure for double-strand breaks [20] as described in detail previously [18]. Cells were labeled for 24 hr with 0.1 μCi/nmol [$^3$H]thymidine followed by washing and incubation for an additional 24 hr in thymidine-free medium prior to incubation with drug and processing as described for the alkaline unwinding assay. Approximately $5 \times 10^5$ cells were lysed on polycarbonate filters (Nucleopore Corp.) with SDS (2%)/EDTA (0.02%). Proteinase K (2 mg/mL) was used to eliminate DNA–protein cross-linking. DNA was eluted using tetrapropylammonium hydroxide at pH 9.6 to 10 at a flow rate of 0.8 mL/hr; fractions were collected over a 17-hr period, and analyzed by scintillation counting. Damage was normalized based on rad equivalence determined by exposure of MCF-7 cells to varied doses of ionizing radiation from a $^{137}$cesium source.

### *DNA Biosynthesis*

Macromolecular biosynthesis was determined by monitoring the rate of incorporation of [$^3$H]thymidine into acid-precipitable material over a time course of 40 min as described previously [18]. MCF-7 cells in 24-well plates (Costar) were exposed to camptothecin for 3 hr and washed with Hanks' buffered salt solution (pH 7.4; Whittaker Biochemicals) at room temperature prior to analysis of [$^3$H]thymidine incorporation into trichloroacetic acid-precipitable material. The percent inhibition of DNA biosynthesis was calculated from the relative rates of incorporation of [$^3$H]thymidine into nucleic acid precursors in drug-treated versus untreated cells.

### *Gene Expression*

After incubation with camptothecin for the indicated times and concentrations, cellular RNA was isolated using the RNA STAT-60 procedure as described by the manufacturer (TEL-TEST "B", INC.). Briefly, approximately $10 \times 10^6$ cells were lysed in 1 mL of RNA STAT-60. The lysate was placed in a 1.5-mL Eppendorf tube, and 0.2 mL of chloroform was added. The mixture was shaken vigorously for 15 sec and allowed to remain for 2–3 min at room temperature. Then the mixture was centrifuged at 12,000 *g* for 15 min at 4°. The upper aqueous phase was removed and placed in a 1.5-mL Eppendorf tube, and 0.5 mL of isopropanol was added to the aqueous phase. This solution was stored for 10 min at room temperature. Following this incubation, the samples were centrifuged for 10 min at 12,000 *g*, the supernatant was removed, and the pellet of RNA was washed once with 1 mL of 75% ethanol and centrifuged for 5 min. Ethanol was aspirated, and the pellet was allowed to air dry before being dissolved in autoclaved milli-Q water. The previously isolated RNA (10 μg) was denatured in 0.02 M of morpholino propane sulfonic acid (pH 7.0), 5 mM of sodium acetate, 1 mM of EDTA, 2.2 M of formaldehyde, and separated in a 1% agarose gel containing 18% formaldehyde [21]. Equal loading of RNA in each lane was confirmed by ethidium bromide staining. Blotting was carried out using Nytran transfer membranes (Schleicher & Schuell). Probes were radiolabeled using a nick-translation kit from Life Technologies and hybridized to blots in the presence of 50 mM sodium phosphate (pH 6.5), 5× Denhardt's solution (0.1% BSA-0.1% Ficoll-0.1% polyvinyl pyrrolidine), 5× SSC (0.75 M of NaCl-0.075 M of sodium citrate), 0.1% SDS, yeast RNA (250 μg/mL), 50% formamide, and 10% dextran sulfate. Hybridizations were for 16–20 hr at 42°. Filters were washed three times at 42° for 5 min in 2× SSC and 0.5% SDS followed by one wash in 2× SSC and 0.5% SDS at 60° and one wash in 0.5× SSC and 0.5% SDS before quantitation of hybridization intensity using a Betascope or by densitometry.

## RESULTS

### *Growth Inhibition by Camptothecin*

The growth inhibitory activity of camptothecin was evaluated using the MTT dye assay [16, 17]. Figure 1A indicates that growth of MCF-7 cells was inhibited in a concentration-dependent manner after acute exposure to camptothecin, with an $IC_{50}$ value of approximately 3 μM.

### *Induction of DNA Damage*

Growth inhibition by drugs such as camptothecin is thought to be a consequence of the induction of DNA damage, resulting from the inhibition of topoisomerase I [1]. DNA damage was determined by alkaline unwinding [19], which assesses combined single- and double-strand breaks and by neutral elution, which monitors double-strand breaks [20]. Figure 1A indicates that bulk damage to DNA was not detectable below a concentration of 5 μM of camptothecin. At 5, 10, and 25 μM of drug, DNA damage increased with increasing concentrations of camptothecin. Figure 1B, which plots growth inhibition by camptothecin versus the induction of DNA strand breaks, indicates that these two functions appear to be unrelated over the lower range of drug concentrations; however, an argument can be made that growth inhibition correlates with the induction of bulk DNA damage at the higher range of camptothecin concentrations (5, 10, and 25 μM).

The induction of double-strand breaks in DNA was assessed by neutral elution, where DNA with double-strand breaks elutes more rapidly than intact DNA. Figure 2 shows

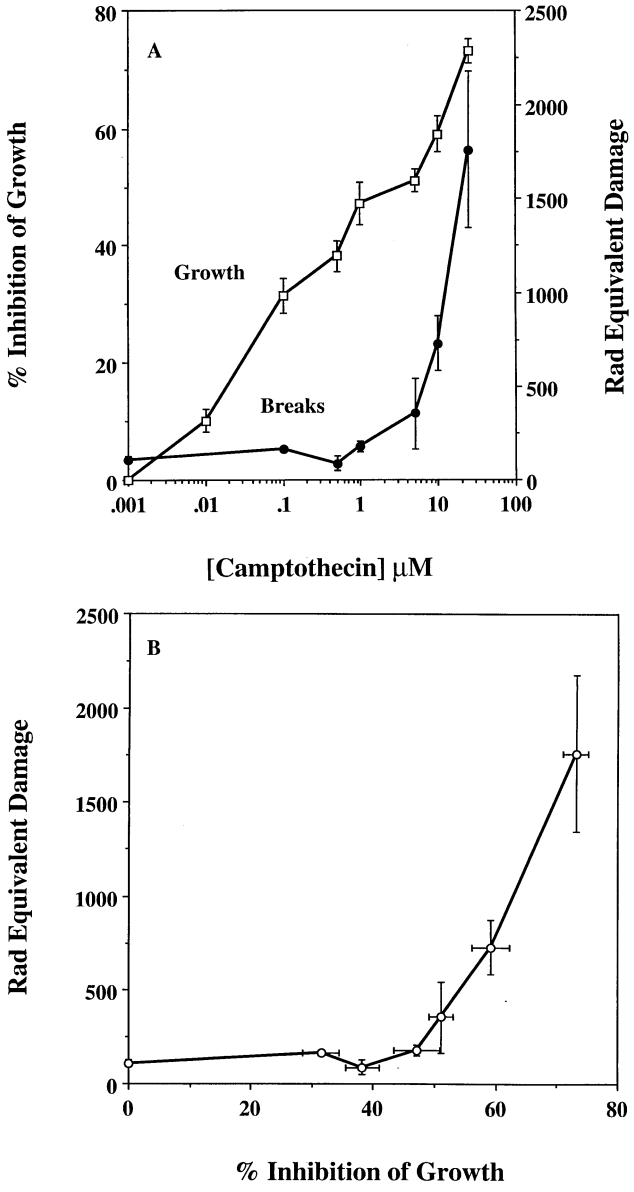


**FIG. 1. (A) Induction of DNA damage and growth inhibition by camptothecin in MCF-7 breast tumor cells. MCF-7 cells were incubated with various concentrations of camptothecin for 3 hr. DNA damage was evaluated by alkaline unwinding, and growth inhibition was monitored using the MTT dye assay. (B) Relationship between induction of DNA strand breaks and growth inhibition by camptothecin. Each value in both panels represents the mean ± SEM for 3 experiments for strand breaks and between 6 and 8 experiments for growth inhibition.**

the rate of elution of DNA from MCF-7 cells treated with camptothecin over the range of 0.1 through 25 μM. These data parallel the findings presented in Fig. 1A, using the alkaline unwinding assay, in that double-strand breaks became apparent only at camptothecin concentrations that exceeded 1 μM.

### *Influence of Camptothecin on c-*myc *Expression*

We have demonstrated previously that topoisomerase II inhibitors, as well as ionizing radiation, suppress expression

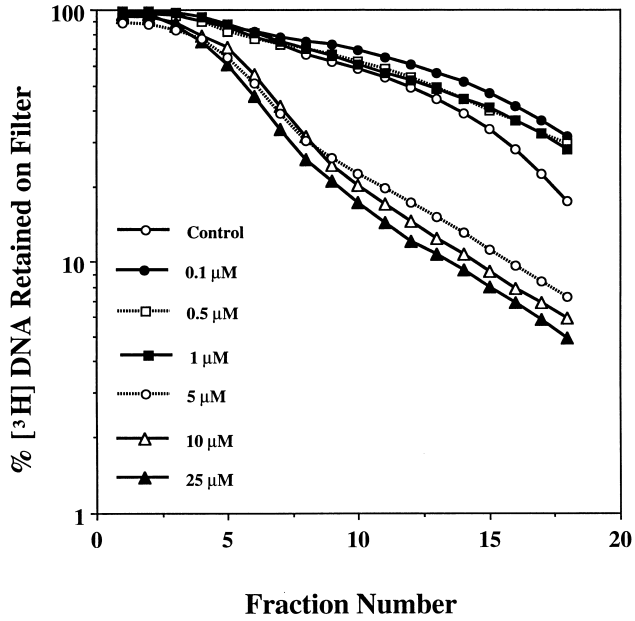


**FIG. 2. Induction of double-strand breaks in MCF-7 cells by camptothecin. MCF-7 cells were incubated with various concentrations of camptothecin (0.1 to 25 μM) for 3 hr prior to assessment of DNA damage by neutral elution. The graph is representative of data from three independent experiments.**

of the c-*myc* gene in MCF-7 breast tumor cells [11–14]. MCF-7 cells were exposed to 10 μM of camptothecin, and c-*myc* expression was monitored over a period of 3 hr. Figure 3 presents a representative northern blot accompanied by pooled data from three experiments, indicating that

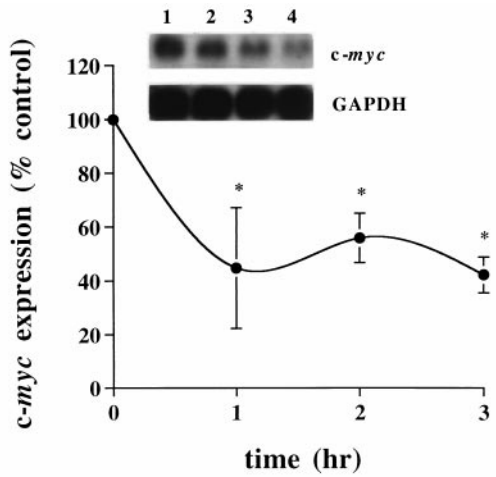


**FIG. 3. Inhibition of c-*myc* expression by camptothecin in MCF-7 breast tumor cells. Inset: Representative northern blot depicting steady-state c-myc and GAPDH mRNA expression after exposure to 10 μM of camptothecin. Lane 1: control at 0 hr. Lanes 2–4: effects on c-*myc* expression of treatment with 10 μM of camptothecin after 1, 2, and 3 hr, respectively. Figure: Summary of pooled data indicating the time-dependent reduction in c-*myc* expression by 10 μM of camptothecin. The ratio of c-myc/GAPDH was utilized for normalization of data and to correct for differences in gel loading. Each point represents the mean ± SEM for 3 replicate experiments. *Significantly different from expression at time 0, with a $P$ value < 0.05.**

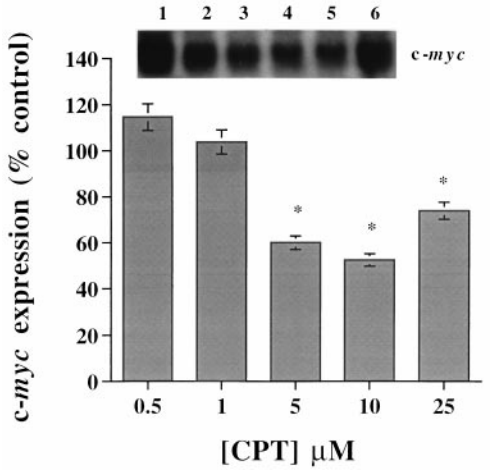


**FIG. 4. Concentration-dependent effects of c-*myc* expression by camptothecin in MCF-7 human breast tumor cells. Inset: Representative northern blot depicting steady-state c-myc mRNA expression after 3 hr. Lane 1: 0.5 μM of camptothecin. Lane 2: 1 μM of camptothecin. Lane 3: 5 μM of camptothecin. Lane 4: 10 μM of camptothecin. Lane 5: 25 μM of camptothecin. Lane 6: control. Figure: The ratio of c-myc/GAPDH expression as a function of the concentration of camptothecin. RNA was isolated at 3 hr after initiation of drug exposure. Each point represents the mean ± SEM for 3 replicate experiments. *Significantly different from controls with a *P* value < 0.05.**

camptothecin inhibited the expression of c-*myc* message and that maximal suppression was observed within approximately 1 hr after drug exposure.

In view of our hypothesis that c-*myc* expression might be linked to the induction of damage to bulk DNA, we evaluated the effects of camptothecin on c-*myc* expression at concentrations that fail to produce detectable damage to bulk DNA (0.5 and 1 μM) and at concentrations associated with the induction of strand breaks in DNA (5, 10, and 25 μM). Figure 4 presents a representative northern blot as well as pooled data from three experiments, demonstrating that camptothecin failed to alter c-*myc* expression at the lower concentrations of drug (where no bulk DNA damage was detected), but that c-*myc* message was reduced by between 30 and 45% at the higher drug concentrations (where DNA strand breaks were evident). In all studies, the expression of GAPDH was essentially unchanged (data not shown), and was used to normalize for drug effects on the expression of c-myc mRNA.

### *Inhibition of DNA Synthesis*

We have reported previously that inhibition of DNA synthesis corresponds with growth inhibition for the topoisomerase II inhibitors [11–13]. The influence of camptothecin on DNA synthesis was evaluated by monitoring the incorporation of [$^3$H]thymidine into acid-precipitable material. Figure 5A demonstrates that camptothecin produced a concentration-dependent inhibition of DNA synthesis. Figure 5B indicates that the inhibition of DNA synthesis, which was assessed 2 hr after drug exposure, closely paralleled growth inhibition (assessed 72 hr after drug exposure) with a correlation coefficient of 0.96.

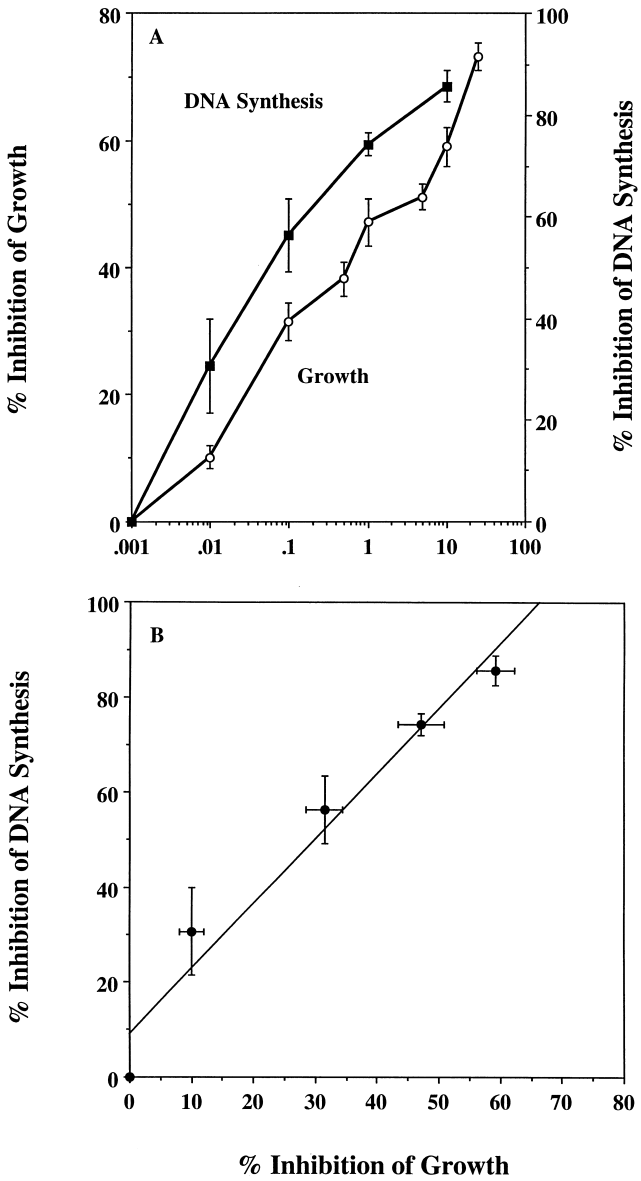


**FIG. 5. (A) Camptothecin-induced inhibition of growth and DNA biosynthesis. MCF-7 cells were incubated with various concentrations of camptothecin for 2 hr and labeled with [$^3$H]thymidine to monitor DNA synthesis. (B) Correlation between inhibition of DNA biosynthesis and growth of MCF-7 cells following exposure to camptothecin. Each value on both graphs represents the mean ± SEM for 3 experiments.**

## DISCUSSION

### *Influence of Camptothecin on DNA Integrity*

Camptothecin induces single-strand breaks in DNA by stabilizing the DNA–topoisomerase I complex, and preventing DNA religation [3–6]. The subsequent induction of double-strand breaks in DNA is thought to occur by interference with progression of the DNA replication fork by the stabilized topoisomerase I–DNA complex [7–9]. In

an early study, Ryan *et al.* [10] demonstrated a correspondence between double-strand breaks in nascent DNA and the antiproliferative activity of camptothecin in SV40-transformed skin fibroblasts. In a subsequent report, hypersensitivity to camptothecin in Cockayne's Syndrome cells was suggested to be related to the persistence of double-strand breaks in nascent DNA [22]. However, more recent findings by these investigators [23] failed to support a correspondence between damage to nascent DNA and loss of clonogenicity in Chinese hamster ovary cells after exposure to camptothecin, as loss of clonogenicity was identical at different levels of DNA damage. Furthermore, O'Connor *et al.* [24] have reported that S-phase population fails to correlate with camptothecin cytotoxicity in human colon carcinoma cells. These findings raise questions regarding the role of damage to nascent (i.e. newly replicated) DNA in the antiproliferative and cytotoxic actions of camptothecin.

The induction of bulk DNA damage by camptothecin (combined single- and double-strand breaks measured by alkaline unwinding and double-strand breaks measured by neutral elution) in MCF-7 cells did not correlate with its growth inhibitory activity. A similar lack of correlation between bulk DNA damage and growth inhibition was reported for the topoisomerase II inhibitors Adriamycin®, VM-26, and *m*-AMSA in MCF-7 cells [11–13]. However, the findings with camptothecin do not necessarily lend themselves to easy interpretation. Since single-strand breaks in DNA induced by camptothecin are rapidly reversible [25], the alkaline unwinding assay may have failed to detect these lesions. Furthermore, the limited sensitivity of the neutral elution assay (approximately 10 Gy equivalents in our hands) would similarly compromise the detection of a small number of double-strand breaks. Finally, despite the caveats stated above, the possibility that nascent double-strand breaks could occur even at the lower concentrations of camptothecin cannot be discounted.

### *Influence of Camptothecin on c-*myc *Expression*

We have suggested previously that signaling events subsequent to DNA strand-break induction/cleavable complex formation could be important for growth arrest in cells exposed to topoisomerase inhibitors or other modalities that induce DNA damage [26, 27]. In this context, Tsao *et al.* [28] as well as Goldwasser *et al.* [29] reported that camptothecin treatment promotes a reduction in $p34^{cdc2}$/cyclin B activity in various experimental cell lines, while Fujimori *et al.* [30] have demonstrated recently an increase in critical protein components of the DNA damage response pathway (p53, gadd 45, and $p21^{waf1/cip1}$) in response to camptothecin in MCF-7 breast tumor cells. The present study demonstrates that camptothecin concentrations that induce bulk damage to DNA also suppress c-*myc* expression. This observation complements our previous findings that topoisomerase II inhibitors and ionizing radiation suppress c-*myc* expression in MCF-7 cells [11–14], and may be relevant to the recent report suggesting that camptothecin activates an S-phase checkpoint [31]. However, in contrast to our previous studies with topoisomerase II inhibitors or ionizing radiation, the suppression of c-*myc* expression by camptothecin was not clearly concentration dependent; furthermore, it is evident that growth inhibition by camptothecin can occur in the absence of suppression of c-*myc* expression.

### *Inhibition of DNA Biosynthesis*

The capacity of camptothecin to inhibit DNA synthesis is well established [32–35]. Our studies demonstrate a close correspondence between the early inhibition of DNA synthesis and the subsequent effects on cell proliferation by camptothecin. The camptothecin-induced inhibition of DNA synthesis could result from interference with progression of the DNA replication fork by the stabilized topoisomerase I–DNA complex [2, 8, 9] or the proposed activation of an S-phase checkpoint [31], i.e. as an early event in the signaling pathway responding to drug-induced DNA damage.

In summary, studies with both the topoisomerase I inhibitor camptothecin and topoisomerase II inhibitors demonstrated a correspondence between the inhibition of DNA synthesis and of growth in MCF-7 breast tumor cells. One evident difference between the effects of the topoisomerase I and topoisomerase II inhibitors in MCF-7 cells is that while VM-26, *m*-AMSA, and doxorubicin all produced suppression of c-*myc* expression that corresponded with growth inhibition, the antiproliferative effects of camptothecin did not appear to be linked to altered c-*myc* expression. Finally, the finding that suppression of c-*myc* expression was observed only at camptothecin concentrations where bulk DNA strand-break induction was evident supports the concept that c-*myc* expression is linked, albeit indirectly, to the DNA damage response pathway in this cell line.

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