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The human cell multiprotein DNA replication complex (MRC): the effect of camptothecin on its ability to support in vitro DNA synthesis

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Abstract *Purpose*: We have previously reported on the isolation and characterization of a multiprotein DNA replication complex (MRC) from HeLa cells that fully supports in vitro DNA replication. Based upon its ability to replicate DNA in a cell-free environment (devoid of other cellular processes) the MRC may serve as a unique model system for investigating the mechanisms of action of anticancer drugs that directly affect DNA synthesis. The experiments described in this report were performed to establish whether the MRC could serve as a model system to examine in detail the mechanism of action of camptothecin, a DNA topoisomerase I inhibitor. *Methods*: We examined the effects of increasing concentrations of camptothecin on HeLa cell survival, intact HeLa cell DNA synthesis and MRC-mediated in vitro DNA replication. We also performed topoisomerase I assays in the presence of increasing concentrations of camptothecin to study the direct effects of the agent on MRC-associated topoisomerase I activity. Furthermore, we employed an SDS precipitation assay to measure the formation of MRC-associated topoisomerase I-cleavable complexes in the presence of increasing concentrations of camptothecin. *Results*: We found a close correlation between the IC_{50} values for intact HeLa cell DNA synthesis (0.15 μM) and MRC-mediated in vitro DNA synthesis (0.05 μM). Similarly, we found that 0.05 μM camptothecin inhibited MRC-associated topoisomerase I activity by approximately 50%. In addition, we found that the formation of MRC-associated topoisomerase I-cleavable complexes increased linearly with increasing concentrations of camptothecin. *Conclusions*: The data presented in this report support the use of the MRC as a model system to study the mechanism of action of camptothecin. We anticipate that future studies with the MRC will help elucidate the cellular consequences of camptothecin-cleavable complex formation.

Key words Camptothecin · Topoisomerase I · DNA replication complexes · Anticancer agents · In vitro model system

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Introduction

Camptothecin is a plant alkaloid that demonstrates potent antitumor activity against a wide range of human cancers [17]. It inhibits both RNA and DNA synthesis in mammalian cells during S phase and causes subsequent G2 cell cycle arrest [18]. Camptothecin possesses a unique mechanism of action in that it traps nuclear topoisomerase I in a reversible enzyme-drug-DNA cleavable complex [5, 9, 11]. In this state, topoisomerase I cannot perform its DNA single strand nicking resealing function required for the relaxation of supercoiled DNA. However, it is unclear whether or not this mode of action is responsible for camptothecin's cytotoxicity in vivo. Researchers speculate that the interaction of drug-stabilized cleavable complexes with advancing replication forks culminates

in replication fork breakage and the accumulation of irreversible topoisomerase I-linked DNA single-strand breaks [6, 18]. These drug-induced recombinational events appear as smears and late-Cairns arcs when the aberrant DNA replication products are resolved on two-dimensional gels [16, 20].

We have utilized a novel in vitro system to examine the inhibition of DNA synthesis and DNA topoisomerase I activity by camptothecin. Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex (MRC) from human cells that supports origin-specific and large T-antigen-dependent simian virus 40 (SV40) DNA replication in vitro [4, 7, 14, 15]. The integrity of the MRC is maintained following treatment with detergents, salt, RNase and DNase, suggesting that the association of the proteins with one another is independent of nonspecific interactions with other cellular macromolecules [24]. We have shown that the DNA replication activity of the human cell MRC is comparable to that observed in intact cells [3]. The proteins observed to copurify with the MRC include the key DNA synthetic enzymes DNA polymerase α, DNA polymerase δ, DNA primase, PCNA, DNA ligase I, DNA helicase, RF-C, RPA, RNase H, DNA topoisomerase II and a fully functional topoisomerase I [3, 15, 24].

In this report, we describe experiments that establish that the MRC could serve as a valuable, in vitro model to examine in detail the mechanism of action of camptothecin. Such work would help elucidate the cellular consequences of cleavable complex formation as well as facilitate the development of improved camptothecin analogues.

Materials and methods

Materials

Camptothecin was purchased from TopoGen, Ohio. The drug was dissolved in dimethyl sulfoxide and stored in aliquots at − 20 °C.

Purified topoisomerase I was also purchased from TopoGen. One unit of enzyme relaxes 0.5 μg DNA in 15 min at 37 °C. The enzyme was supplied at a concentration of 2 units/μl, 4 ng/μl.

α^{32}P-dCTP (3000 Ci/mmol; 370 MBq/ml; 10 mCi/ml) and ^{3}H-thymidine (90 Ci/mmol; 37 MBq; 2.5 mCi/ml) were obtained from DuPont New England Nuclear.

Cell culture and harvest

Suspension cultures of HeLa cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml of medium) were harvested and washed three times with phosphate-buffered saline: 8.4 m*M* Na_2HPO_4, 137 m*M* NaCl, 1.5 m*M* KH_2PO_4. The cells were then pelleted by low-speed centrifugation (200 g, 5 min., 4 °C). Cell pellets were stored at − 80 °C prior to initiating isolation of the MRC.

Fractionation and chromatographic scheme for isolation of HeLa cell MRC

The HeLa cell MRC was isolated as described by Malkas et al. [15]. The protein fraction designated Q-Sepharose peak (1 μg/μl), which contains the DNA replication-competent MRC, was used in the experiments described in this report.

HeLa cell survival assays

HeLa cell survival assays were performed in the presence of increasing concentrations of camptothecin, according to the procedure described by Ryan et al. [18].

Measurement of intact HeLa cell DNA synthesis

Exponentially growing HeLa cells were incubated at 37 °C with increasing concentrations of camptothecin in the presence of ^{3}H-thymidine. After a 1-h incubation, cells were lysed [8] and the amount of radiolabel incorporated into DNA was determined by the isolation and counting of acid-insoluble material [19].

In vitro SV40 DNA replication assay

Assay reaction mixtures (25 μl) contained 80 m*M* Tris-HCl, pH 7.5; 7 m*M* $MgCl_2$; 1 m*M* DTT; 1–5 μg Q-Sepharose peak; 0.5–1.0 μg purified SV40 large T-antigen; 25 ng plasmid pSVO + containing an insert of SV40 replication-origin DNA sequences [21]; 100 μ*M* each dTTP, dATP, dGTP; 200 μ*M* each rCTP, rGTP, rUTP; 4 m*M* ATP; 50 μ*M* α^{32}P-dCTP; 40 m*M* creatine phosphate; and 1 μg creatine kinase. The standard reaction, conducted in the absence or presence of camptothecin, was incubated for 2 h at 37 °C. Replication assay products were processed using DE81 filter binding to determine the amount of radiolabel incorporated into acid-insoluble material [19].

Topoisomerase I assay

Topoisomerase I activity was measured by incubating 0.3 μg supercoiled pSVO + DNA with either 8 μg Q-Sepharose peak, in the absence or presence of increasing concentrations of camptothecin, or 2 units purified topoisomerase I. Incubations were performed for 20 min at 37 °C. Each reaction (15 μl) was stopped with 1% SDS and the DNA products resolved on a 1.0% agarose gel containing TAE buffer (40 m*M* Tris acetate, 2 m*M* EDTA). After ethidium bromide (1 μg/ml) staining of the gels [19], topoisomers were visualized with an ultraviolet light source.

Quantitation of the amount of topoisomerase I present in the Q-Sepharose peak

The densities of fully converted form II DNAs produced by purified topoisomerase I (5 units) and Q-Sepharose peak (3 μl) were scanned by a laser densitometer. The form II DNA relaxed by the purified enzyme possessed a twofold greater density than that relaxed by the MRC-associated enzyme. Based upon this comparison, a mathematical relationship was used to estimate the amount of topoisomerase I present in the Q-Sepharose peak:

$$\text{topo I in Q-Seph peak (ng/μl)} = \frac{0.5\ (10\ \text{ng purified topo I})}{3\ \mu\text{l Q-Seph peak}}$$

$$= 1.66\ \text{ng/μl}$$

5′ end-labeling of linear plasmid DNA

Plasmid pSVO + DNA (20 μg) was digested with 40 units Hind III for 60 min at 35 °C. The digested DNA (1 μg) was incubated with 1 unit of Klenow fragment (5 units/μl) for 30 min at room temperature in a reaction mixture containing: 2.5 μl end-labeling buffer (0.5 *M* Tris-HCl (pH 7.6), 0.1 *M* $MgSO_4$, 1 m*M* dithiothreitol, 500 μg/ml BSA); 0.75 μl 100 m*M* $MgCl_2$; 2.5 μl 10X dGTP; 2.5 μl 10X dATP; 2.5 μl 10X dTTP; 4 μl ^{32}P-dCTP (3000 Ci/mmol, 10 mCi/ml); 2.25 μl dH_2O. Reactions were stopped by the addition of 1 μl 0.5 *M* EDTA and heating to 65 °C for 5 min; the reaction mixtures were then diluted to 100 μl with a buffer containing 0.1 *M* NaCl, 10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA. Unincorporated deoxynucleotide triphosphates were removed by chromatography through a p60 gel filtration column [19].

SDS precipitation of topoisomerase I-cleavable complexes

Cleavage of DNA by human topoisomerase I was performed as follows. Briefly, 5 units purified topoisomerase I or 8 μg Q-Sepharose peak, 1 μl end-labeled pSVO + DNA and increasing concentrations of camptothecin were added to a reaction buffer containing 10 m*M* Tris-HCl (pH 9.0), 0.5 m*M* EDTA and 10 μg/ml BSA. After a 5-min incubation at 37 °C, SDS precipitation of double-stranded DNA topoisomerase I complexes was performed as described by Liu et al. [13].

Results

Inhibition of HeLa cell survival, intact HeLa cell DNA synthesis, and MRC-mediated DNA synthesis by camptothecin

We performed HeLa cell survival assays (Materials and methods) to verify that camptothecin affects the ability of mammalian cells to proliferate, as previously reported [1]. We exposed HeLa cells to increasing concentrations of camptothecin for a 1h, then washed the cells and incubated them in fresh drug-free medium for of 8–10 days. Figure 1 shows that exposing HeLa cells to increasing concentrations of camptothecin for 1 h, impaired cell survival. At the highest concentration of camptothecin used in this experiment (5 μ*M*), approximately 66% of the HeLa cells remained viable. This result is indicative of the S-phase specificity of the drug and is consistent with previous reports on camptothecin cytotoxicity [22].

Topoisomerase I plays a pivotal role in DNA replication, facilitating replication fork migration by unwinding positive supercoils as they accumulate ahead of the fork. The necessity for topoisomerase I in DNA synthesis is underscored by the sensitivity of intact HeLa cell DNA synthesis to low concentrations of camptothecin. Figure 2 shows that ^{3}H-thymidine incorporation into DNA by exponentially growing HeLa cells (Materials and methods) is inhibited by 50% at approximately 0.15 μ*M* camptothecin–a result consistent with previously published results on camptothecin's inhibition of nucleic acid synthesis in HL60 cells [12]. The occurrence of low levels of DNA replication at higher drug concentrations suggests that topoisomerase II may partially substitute for the function normally provided by topoisomerase I [2].

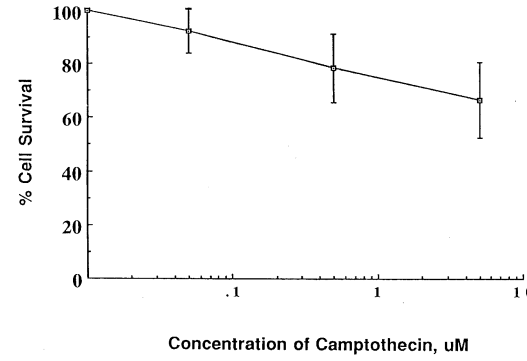

Fig. 1 Effect of camptothecin on HeLa cell survival. HeLa cells (500) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. Cells were then exposed for 1 h to increasing concentrations of camptothecin (0, 0.05, 0.5, 5 μ*M*) dissolved in DMSO. Control plates contained DMSO alone. The number of colonies formed in the presence of DMSO were within 5% of those formed in the absence of DMSO. After removal of drug from the cell cultures, the cells were rinsed twice with warm phosphate-buffered saline and then incubated in fresh drug-free medium for an additional 8–10 days. Colonies were visualized by staining with trypan blue dye. This graph represents the average of three independent experiments; bars represent standard deviations from the average. For the point apparently lacking error bars, the deviation from the average was contained within the symbol

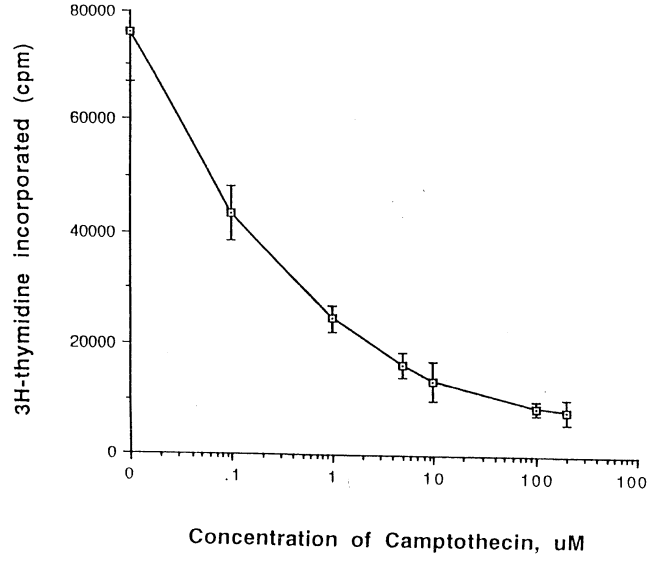

Fig. 2 Effect of camptothecin on intact HeLa cell DNA synthesis. HeLa cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were then labeled with ^{3}H-thymidine (1 μCi/ml of medium) and exposed to increasing concentrations of camptothecin (0, 0.1, 1, 5, 10, 20, 100 μ*M*) dissolved in DMSO. Plates containing DMSO alone served as controls. After a 1-h incubation, cells were lysed and the level of DNA synthesis was measured by the isolation and counting of ^{3}H in acid-insoluble material. This graph represents the average of four separate experiments; error bars represent standard error of the means

Table 1 Effect of camptothecin on MRC-meditated SV40 DNA replication in vitro. MRC-driven in vitro SV40 DNA replication assays were performed in the presence of increasing concentrations of camptothecin (0, 0.05, 0.5, 5, 50 μ*M*) dissolved in DMSO. Reactions containing DMSO alone served as a control. The amount of ^{32}P-dCTP incorporated into DNA replication assay products was determined using DE81 filter binding

Camptothecin concentration (μ*M*)	^{32}P-dCTP incorporation into DNA (open)	Inhibition (%)
0	36 387	0
0.05	15 049	59
0.5	14 315	61
5	11 343	69
50	3 822	89

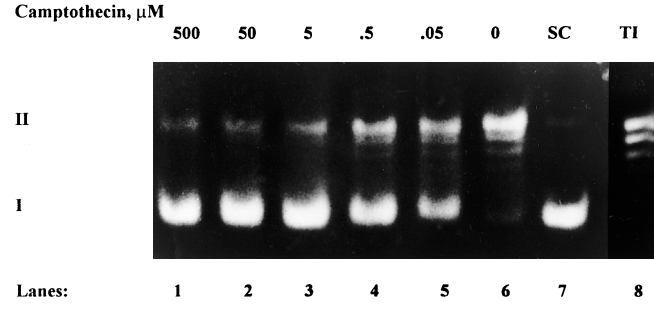

Fig. 3 Inhibition of MRC-associated topoisomerase I activity by camptothecin. Reaction assays containing 8 μg of the Q-Sepharose peak were incubated for 15 min at 37 °C with 0.3 μg supercoiled pSVO+, in the presence of increasing concentrations of camptothecin (0, 0.05, 0.5, 5, 50, 500 μ*M*) dissolved in DMSO. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (1 μg/ml) staining of the gels, topoisomers were visualized by illuminating gels with an ultraviolet light. Lane 7 shows the position of supercoiled pSVO+ DNA in the presence of DMSO (*lane 6* conversion of supercoiled DNA to relaxed open circle form II DNA by the MRC-associated topoisomerase I present in the Q-Sepharose peak, *lanes 1–5* inhibition of MRC-topoisomerase I activity by increasing concentrations of camptothecin, *lane 8* conversion of supercoiled DNA to relaxed form II DNA by 2 units of purified topoisomerase I)

Similarly, a low concentration of camptothecin was found to inhibit the ability of the MRC to support in vitro DNA replication. In vitro SV40 DNA replication assays (Materials and methods) incubated with increasing concentrations of camptothecin were analyzed for total ^{32}P-dCTP incorporation into DNA. MRC-mediated DNA replication was inhibited by almost 60% when reactions contained 0.05 μ*M* camptothecin (Table 1). The results presented in Table 1 are typical of those obtained in this type of experiment. The relatively close correlation between the concentrations of camptothecin inhibiting DNA synthesis in intact cells and in the MRC-driven in vitro reactions supports the potential role of the MRC as a meaningful model to study camptothecin and other anticancer agents that directly affect DNA synthesis.

Effect of camptothecin on MRC-associated topoisomerase I activity

We performed topoisomerase I assays to establish that the topoisomerase I activity present in the MRC is fully able to produce the hallmark ladder of DNA intermediates while converting a form I supercoiled plasmid DNA into a form II DNA (Fig. 3, lane 6). The pattern of topoisomers produced by the MRC-associated topoisomerase I was indistinguishable from that generated by the purified enzyme (Fig. 3, lanes 6 and 8). Furthermore, we assessed the level of topoisomerase I activity present in the Q-Sepharose peak by laser densitometry to quantitate the densities of fully converted form II DNAs produced by the MRC-associated and purified enzymes (data not shown). We found that the form II DNA generated by the purified topoisomerase I enzyme possessed a twofold greater density than that generated by the MRC-associated enzyme. Therefore, we estimated that the level of topoisomerase I present in the Q-Sepharose peak was approximately 1.66 ng/μl (Materials and methods).

In order to examine the effect of camptothecin on MRC-topoisomerase I activity, we performed topoisomerase I assays in the presence of increasing concentrations of the drug (Materials and methods). In these assays, inhibition of topoisomerase I activity by camptothecin resulted in the accumulation of form I DNA. We observed an extensive level of inhibition of the MRC-topoisomerase I activity by camptothecin (Fig. 3, lanes 1–5); as little as 0.05 μ*M* camptothecin caused a significant retention of form I DNA. From these experiments, we determined that 0.05 μ*M* camptothecin inhibited MRC-topoisomerase I activity by over 50%. This concentration of camptothecin is comparable to that which inhibits both in vitro and intact cell DNA synthesis by 50%, supporting the premise that the inhibition of DNA replication by camptothecin results from the drug's inhibition of topoisomerase I activity.

SDS precipitation of camptothecin-stabilized cleavable complexes

Camptothecin inhibits topoisomerase I activity by trapping the enzyme in a reversible enzyme-drug-DNA cleavable complex, the formation of which leads to replication fork arrest and DNA fragmentation [22]. Studies have revealed that precipitation of the cleavable complex by protein denaturants yields DNA single-strand breaks and results in the covalent linkage of topoisomerase I to the 3′ end of the breaks via a phosphotyrosine bond [10]. Utilizing 5′ end-labeled substrate DNA, the relative amount of cleavable complexes stabilized by camptothecin can be quantified via

the isolation and counting of radioactive topoisomerase I-linked DNA breaks [13]. Figure 4 shows the precipitation of purified and MRC-associated topoisomerase I DNA cleavable complexes in the presence of increasing concentrations of camptothecin (Materials and methods). The formation of drug-stabilized purified topoisomerase I-cleavable complexes leveled off at 0.5 μM camptothecin (Fig. 4A), while the formation of drug-stabilized MRC-associated topoisomerase I-cleavable complexes increased linearly with increasing amounts of drug (Fig. 4B). This is a consequence of the greater amount of MRC-associated topoisomerase I used in these assays compared to the purified enzyme. Overall, the increase in MRC-topoisomerase I cleavable complex formation at higher concentrations of camptothecin correlates with our observation regarding the loss of MRC-associated topoisomerase I activity at higher concentrations of camptothecin.

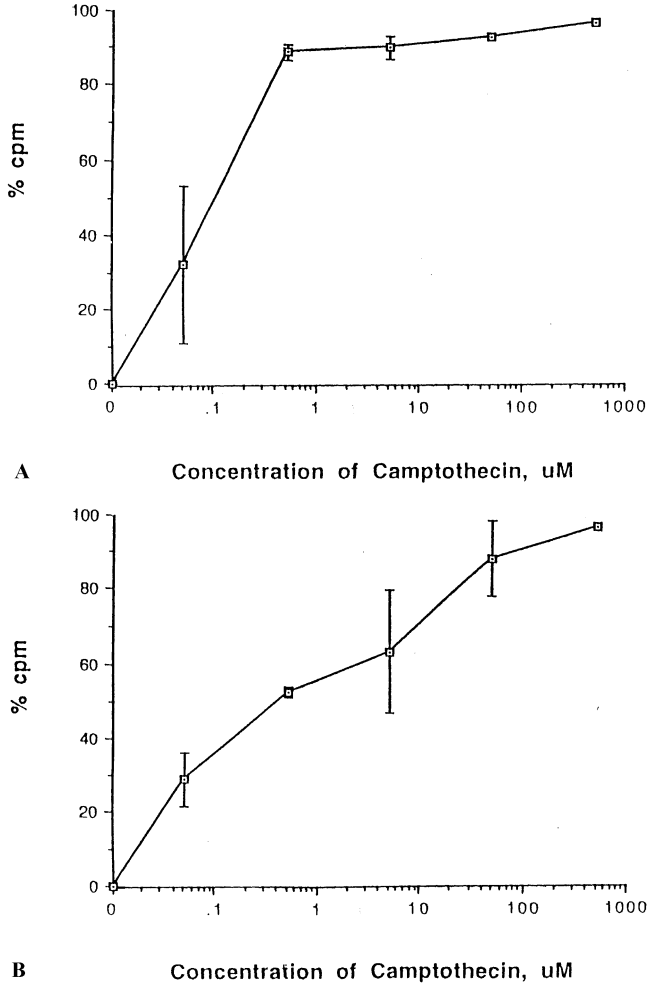

Fig. 4 **A, B** SDS precipitation of camptothecin-topoisomerase I cleavable complexes. **A** Reaction assays containing 5 units of purified topoisomerase I and 1 ng of 5′ end-labeled pSVO+ DNA were incubated for 5 min at 37 °C with increasing concentrations of camptothecin (0, 0.05, 0.5, 5, 50, 500 μM) dissolved in DMSO. Reactions were stopped by the addition of a solution containing 2% SDS, 2 mM EDTA and 0.5 mg/ml sheared salmon sperm DNA [12]. After SDS precipitation of topoisomerase I-DNA cleavable complexes, pellets were transferred to vials containing scintillation cocktail and counted. The data presented are the average of two independent experiments; error bars represent the standard deviations from the average. **B** SDS precipitation assays were performed as described for A using 8 μg MRC. The data are the average of three independent experiments; error bars represent the standard error of the mean

Discussion

Although human cell-derived DNA polymerases α and δ, RP-A, RF-C, topoisomerases I and II, and PCNA have been identified as playing roles in SV40 DNA replication in vitro, their functional organization allowing for the efficient replication of DNA in vivo has not been well defined. Over the past several years, a number of researchers have reported on the roles that multiprotein complexes play in eukaryotic cellular DNA replication [14]. Our laboratory was the first to describe an MRC isolated from human cells that faithfully duplicates DNA in the presence of large T-antigen and SV40 viral origin sequences [3, 7, 15]. In addition to identifying many of the key DNA replication enzymes that comprise the MRC, we have performed experiments that suggest that the MRC replicates DNA in vitro in a manner similar to that observed in intact cells [3, 15, 24].

We believe that the MRC will prove to be a powerful in vitro system to study the mechanisms of action of anticancer agents that directly affect DNA synthesis. First, as discussed earlier in this report, the DNA replication activity of the MRC is as sensitive to camptothecin poisoning as intact cell DNA synthesis. In another study, in which investigators examined the effect of camptothecin on SV40 DNA replication mediated by HeLa cell extracts, it was found that much higher concentrations of camptothecin are required to inhibit DNA synthesis as compared to its effect with intact cells [23]. This result suggests that the DNA replication activity of the crude HeLa cell extracts is not especially sensitive to the action of camptothecin. It also provides support for the use of the MRC as an appropriate system to study camptothecin's mechanism of action. Secondly, MRC-intact topoisomerase I activity was inhibited by 50% at 0.05 μM camptothecin. This concentration correlates with those required to inhibit in vitro and intact cell DNA replication by 50%, supporting the premise that camptothecin inhibits nucleic acid synthesis by selectively targeting topoisomerase I. Finally, the increased formation of MRC-intact topoisomerase I-DNA cleavable complexes at higher concentrations of camptothecin is consistent with the more pronounced inhibition of MRC-intact topoisomerase I activity by higher concentrations of the drug. Our results suggest that camptothecin interacts with the intact cell topoisomerase

I and the MRC-associated topoisomerase I in a similar manner.

Several aspects of camptothecin cytotoxicity remain to be investigated; for example, uncovering the lethal cellular events that occur beyond cleavable complex formation. We believe that some of these questions can be answered by employing the MRC as a novel system to study the effects of camptothecin on DNA replication. We anticipate that future studies with the MRC will facilitate a greater understanding of camptothecin's mechanism of action and contribute pertinent information required for the development of analogues with improved anticancer activity.

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